THE CHEMICAL ESTIMATION OF ADRENALINE, AND THE USE OF THE ETHYLENE DIAMINE CONDENSATION METHOD FOR THE QUANTITATIVE ESTIMATION OF PLASMA CONCENTRATIONS OF ADRENALINE IN PERIPHERAL VENOUS BLOOD DURING INSULIN HYPOGLYCAEMIA

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P R E F A C E

The practice of anaesthesia, potentially a highly scientific branch of modern medicine, affords continual stimulation to the clinical anaesthetist interested in applied pharmacology. It is an understatement to assert that a close association between the pharmacologist and the anaesthetist is desirable. The studies to be described in this thesis may appear to have little direct connection with the pharmacology of anaesthetic drugs; they were performed, however, in the course of a general evaluation of chemical methods for the estimation of adrenaline in blood, in an endeavour to find a suitable technique which could be established and employed for basic and clinical research on the mode of action of drugs used in general anaesthesia.

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C O N T E N T S

1 All statistical data was calculated according to information contained in "Statistical Methods" G.W. Snedecor (1940), Iowa State College Press.

2 These explanatory notes are referred to in the text by bracketed numbers. Note (29) concerns biological assay.

A CONSIDERATION OF CHEMICAL METHODS FOR THE ESTIMATION OF ADRENALINE

In 1856, Vulpian described the formation of a green colour when the adrenal medulla was moistened with ferric chloride; iodine produced a red coloration. The medulla was shown to develop a blue colour with ferric sulphate (Colin, 1856), while the formation of a brown colour on adding dichromate to adrenal extracts was described by Henle in 1865. These observations laid the foundation for subsequent attempts to estimate adrenaline by chemical means.

Following the isolation of the physiologically active principle of the adrenal medulla, by Takamine and by Aldrich (1901), attempts were made to utilise various colour reactions for the estimation of adrenaline in medullary extracts:

(1) Iodine reaction (rose-red) suggested by Abelous, Soulié and Toujan (1905).

(2) Fränkel-Albers reaction, employing potassium bi-iodate and phosphoric acid (1909). The same reddish-violet colour was produced by the action of iodic acid (Krauss, 1908).

(3) Comessatti's reaction (red) using mercuric chloride (1909).

(4) Potassium persulphate reaction (Ewins, 1910).

Ewins (1910), in an appraisal of the above methods, pointed out that the colour reactions were wholly or in part dependent on the oxidation of adrenaline. This was also the basis of the methods employing manganese dioxide (Zanfrognini, 1909), gold chloride (Gautier, 1912), and potassium ferricyanide (Cevidalli, 1909); indeed, it soon became obvious that adrenaline could be oxidised by most of the common oxidising agents, the red oxidation product then being estimated colorimetrically. The typical "chromaffin" staining reaction (Henle, 1865), with dichromate, has more recently been shown to be due to the direct oxidation of adrenaline or other related aromatic phenol derivatives (Bennett, 1941). Furthermore, the reaction does not require dichromate - various oxidising salts will produce a similar colour (Gerard et al. 1930).

In a comprehensive review of the colorimetric tests for the estimation of adrenaline Barker, Eastland and Evers (1932) classified the methods described in the literature into three groups:

(1) Those dependent primarily on the formation of a red coloured oxidation product:

Ferric chloride reaction
Potassium iodate reaction, and its modifications.
Potassium persulphate reaction.
Mercuric chloride reaction.
Reaction with other oxidising agents - hydrogen peroxide, potassium ferricyanide, potassium permanganate, potassium dichromate, sodium bismuthate, sodium hypobromite, manganese dioxide, and the halogens.

(2) Those dependent on the presence of the catechol grouping:

Folin's reagent - phosphotungstic acid.
Ammonium molybdate reagent.

(3) A miscellaneous group:

Diazobenzenesulphonic acid reaction.
Azzolini's test (1931).
Orru's test - ninhydrin (1929).
Reaction with ferrous sulphate and sodium phosphate.

Miscellaneous group

The diazobenzene sulphonic acid reaction was described by Friend (1923) for quantitative estimation of adrenaline in urine, and it was used by Sansoni (1930), who considered it sensitive and easy to apply. Barker et al., however, found no definite red coloration either with pure adrenaline or with urine to which adrenaline had been added. The tests described by Azzolini (1931), Orru (1929), and Paget (1931) were found to be so unspecific that they were unreliable even in qualitative tests. The ferrous sulphate-sodium phosphate reaction was too insensitive.

Ferric chloride reaction

This is characterized by the formation of a transient grass-green coloration, changing to red on standing, by careful addition of alkali or, most rapidly, by boiling. However, Barker et al. (1932) stated that ferric chloride solution alone gave a similar colour on boiling. Also, adrenal cortical extracts which contained no adrenaline gave duplicate reactions with this test.

Potassium iodate reaction

This was applied quantitatively by Hale and Seidell

(1911), who correlated the results obtained using iodate with those obtained by biological assay (pressor response). They found the activity as determined by chemical assay to be 30 per cent lower. In spite of this, they considered the test suitable for assay of adrenal extracts. The iodate reaction was also studied by Scoville (1920). An orange-red colour was produced in neutral solution, and a reddish-violet colour in the presence of acetic or hydrochloric acid. According to Barker et al. (1932), the method compared favourably with the biological methods provided the acidity of the original solution was known and reducing substances such as bisulphite were absent. Concentrations above 1/100,000 could be estimated, and it was comparable to the persulphate test which, however, was easier to perform.

Potassium persulphate reaction

Ewins (1910), had described this test as the most sensitive for adrenaline, claiming a sensitivity of 1/5,000,000, but Barker et al. (1932), found the lower limit to be 1/100,000, and they investigated the test in detail, finding it to be the most specific for adrenaline of all the tests they examined. It appeared to depend on the presence of a substance containing a benzene ring with an OH group in the para-position on the ring and a primary or secondary amino-group on the side-chain.(1) Thus, Ewins (1910) found the following bases allied to adrenaline would give a positive persulphate reaction - epinine; noradrenaline; dihydroxyphenylethylamine (2). Oxidation products such as amino-aceto catechol (3) and its derivatives did not give the reaction. Although not as sensitive as Folin's

test, the persulphate reaction was considered by Barker et al., to be sufficiently accurate to be used for the determination of adrenaline in adrenal glands and for other similar purposes.

Mercuric chloride reaction

This was originally suggested by Comessatti (1909) and modified by Ewins (1910). Its application to adrenal gland extracts was reported by Bailly (1924), and by Paget and Loheac (1928). It was further modified by Stuber, Russmann and Proebsting (1923a) who claimed a sensitivity of 1/100,000,000, by adding to adrenaline solutions a mixture of sulphanilic acid, potassium iodate and mercuric chloride, then boiling. However, Barker et al., did not find this test to be sufficiently sensitive. They also found that the reagents themselves produced an orange brown colour on boiling.

Amonium molybdate reaction

This test was described by Rae (1930), and depends on the catechol grouping in the adrenaline molecule, a yellow colour being produced. Although not as sensitive as the persulphate reaction, the test could be used for pure solutions down to a concentration of about 1/20,000. It was found by Barker et al., to give misleading results for adrenal gland extracts, and it could not be applied to urine because of the presence of other phenolic substances.

Phosphotungstic acid reaction

Folin's reagent was described for the determination of uric acid (Folin and Denis, 1913), but its sensitivity for adrenaline was found to be three times that for

uric acid. However, ascorbic acid, glutathione, and most easily oxidisable substances gave some blue colour, especially the aromatic polyhydroxy-compounds (4). The test was applied to adrenaline by Folin et al. (1913), by Maiweg (1922) and by Barker and Marrian (1927), but Barker et al., found that the method gave too high results when applied to adrenal extracts. They modified the test by using sodium hydroxide in place of sodium carbonate, the increased alkalinity enhancing its reliability; with pure solutions a straight line was obtained for the colour concentration curve.

The conclusions drawn by Barker et al. (1932), from their study of the available colorimetric tests for adrenaline, were as follows:

> The potassium persulphate test, although not as sensitive as the phosphotungstic acid test of Folin, gave results comparable with those observed by biological assay when the necessary conditions, including control of temperature and pH were observed (5). The potassium iodate reaction also appeared satisfactory but was less extensively studied. The ferric chloride, ammonium molybdate and phosphotungstic acid tests were non-specific. The latter test was extremely sensitive however and gave good results with comparatively pure adrenaline solutions. The other tests were unsatisfactory even from a qualitative point of view.

Colorimetric estimation of adrenaline in blood

Battelli (1902a) appears to have been the first to attempt an estimate of the adrenaline content of blood by means of a chemical method. He used the ferric chloride reagent, which was both insensitive and unspecific since bilirubin and other substances present in blood give the same colour as adrenaline; the reaction is also influenced by pH (Pekkarinen, 1948). Wiesel and Schur (1907) also used the ferric chloride test.

In 1913, Folin, Cannon and Denis used their phosphotungstic acid method for blood adrenaline estimations, and they affirmed: "It is worthy of note that incidental experiments have proved that the method here described is sufficiently delicate, when applied to blood taken from the adrenal veins, to give a stronger colour reaction after stimulation of the splanchnic nerves than before." The same method was also used by Autenrieth and Quantmeyer (1921).

However, Johannessohn (1916) considered that both oxidising and reducing methods for the chemical estimation of adrenaline were only suitable for pure solutions.

The problem of estimating adrenaline in blood was one of considerable difficulty, because of the presence of interfering substances which were capable of producing false positive results in the available colorimetric tests. Ascorbic acid, glutathione, various polyhydroxy compounds, ergothione, uric acid and indeed many reducing substances were possible contaminants (6).

In 1923, an important step toward the effective separation of adrenaline from biological extracts prior to colorimetric estimation, was taken by Whitehorn, who described the use of "permutit" for this purpose. Two years previously, Whitehorn had observed that adrenaline could be completely removed from solution by gentle shaking with "permutit." "Permutit," the trade name for a synthetic zeolite (or alumino-silicate) of the approximate composition 2 SiO_2, Al_2O_3, $Na_2O.6\ H_2O$, is used industrially for softening water and refining sugar. Whitehorn found that all the substances removed by "permutit" were relatively strong bases (with a basic dissociation constant of 5×10^{-9} or greater (7)) and it could be used to separate relatively strong nitrogen bases from weaker nitrogen bases or non-basic substances. "Permutit" removed alkalylamines (8); the strongly basic amino-acids, e.g. lysine, arginine and histidine were also removed. The reaction was reversible, occurring by a process of "trading bases" and obeying the "Law of Mass Action" (9). According to Whitehorn, the following substances were not taken up by "permutit" - urea, uric acid, the amino-acids creatine, creatinine, non-basic polyphenol compounds (10). The ability of "permutit" to remove bases from solution was found to be adversely affected at low pH levels.

Although Whitehorn used "permutit" for separation of adrenaline from blood, subsequently employing phospho-tungstate reagent for colorimetric estimation, he was justifiably sceptical about the values obtained - 1.5 mgm.

adrenaline per litre in human blood.

In 1935, Whitehorn developed an improved chemical method for the estimation of adrenaline in blood. This was based on two principles:

(1) The use of a column of silicic acid, through which the deproteinised blood filtrate was passed, for the separation of adrenaline from other reducing substances prior to colorimetric estimation. This also served to concentrate the adrenaline - 40 per cent of the adrenaline present in 50 ml. blood filtrate was concentrated into 5 ml. (This required the use of a correction factor). The adsorbed adrenaline was eluted from the silicic acid column by means of sulphuric acid.

(2) Utilisation of a sensitive arsenomolybdic acid reagent for the determination, the reagent being reduced by adrenaline to give a blue colour.

Whitehorn noted that the arsenomolybdic acid reagent was not specific for adrenaline. The trioses, glyceraldehyde and dihydroxyacetone, and the diose glycolaldehyde, gave a strong reaction under the acid conditions specified (11). There was no reduction with hexoses or pentoses (12). Reduced glutathione, in the concentrations found in blood, gave a marked blue colour. However, this and non-basic reducing substances, and even the strongly basic alkaloid ergothioneine, were allegedly excluded by the use of silicic acid for adsorption of adrenaline (13). Organic substances reducing the arsenomolybdic reagent could be grouped into thiols and

enediols (14). Monophenols, resorcinol, and uric acid did not reduce the reagent (15).

Whitehorn stated "that the procedure devised to insure specificity for adrenaline does accomplish that purpose is perhaps best indicated by my uniform failure to find by this method demonstrable quantities of adrenaline in human venous blood." Serious errors resulted, however, if the blood was not treated with trichloroacetic acid within one minute of venipuncture. This was later confirmed by Shaw (1938). If the blood was allowed to stand for 10 to 15 minutes, the apparent adrenaline rose as high as 120 µg. per litre. Whitehorn also noted that small quantities of adrenaline added to blood could be determined with lower losses than when simply diluted with distilled water. In recovery experiments, Whitehorn recovered 84 to 107 per cent of added adrenaline. The limit of sensitivity was 1/50,000,000.

The method of Whitehorn was employed by Dopy and Weisinger (1938), but it was adversely criticised by Eichler and Noack (1939) on the grounds that adsorption of adrenaline on silicic acid was inefficient. However, Kobro (1946), using a slightly modified version of Whitehorn's method (1935), found average values of 40 to 50 µg. adrenaline per litre in peripheral venous blood. Giordano and Zeglio (1938, 1939) also modified the method of Whitehorn by taking blood (or other solution containing adrenaline) into sodium thiosulphate to prevent oxidation; however, they found excessively high values of adrenaline in blood.

Barker et al. (1932), during their review of the colorimetric methods for the estimation of adrenaline, stated briefly that they found no adrenaline in the filtrate obtained from the trichloroacetic acid precipitation of defibrinated blood, using the persulphate test for estimation. Viale (1933), using a modified mercuric chloride reagent, estimated the normal adrenaline content of dog blood to be about 2.5 mg. per litre. Kobayashi (1935), using phosphotungstate reagent, obtained values of about 70 µg. adrenaline per litre in rabbit blood. Until 1938, when Shaw described his modification of the method of Whitehorn, the chemical methods for the estimation of adrenaline in blood appear to have been quite unspecific.

Shaw demonstrated in 1938 that preliminary treatment of adrenaline with alkali, in the presence of oxygen, increased the colour formed with arsenomolybdic acid reagent. He stated: "adrenaline is known to be oxidised under these conditions, and apparently one of the products of oxidation is a more active reducing agent than adrenaline itself; this product is itself destroyed by longer exposure to alkali. The nature of this substance is unknown." A series of substances allied to adrenaline were tested, but none produced as much colour as adrenaline (page 96). p-Sympatol was the only substance which resembled adrenaline in showing an increased colour after treatment with alkali.

Shaw concluded that if the reducing power of a tissue extract was increased by treatment with alkali

the observation could be taken as evidence that the extract contained adrenaline or some other phenol with a similar side chain. As Whitehorn had stated earlier, a number of substances other than phenols reduced the arsenomolybdic acid reagent; in the list of interfering substances Shaw included (the enediol) ascorbic acid and the alkaloid eserine (which contains a benzene ring) but he stated that these interfering substances were not adsorbed by the aluminium hydroxide used for the separation of catechol amines (16).

Shaw utilised specially prepared aluminium hydroxide for the adsorption of adrenaline. To avoid the use of a correction factor, the aluminium hydroxide was afterwards dissolved in the reagents of the test, so that it was hoped to recover adrenaline completely. The optimum pH for adsorption was found to be 8 to 8.5. On the acid side of pH 6 there appeared to be no adsorption of adrenaline. The deproteinised blood extract was first shaken with aluminium hydroxide at pH 4 to remove glutathione and other possible interfering substances. According to Shaw, ascorbic acid was not adsorbed at either acid or alkaline pH. After shaking with aluminium hydroxide at pH 4, the supernatant was retreated with aluminium hydroxide at pH 8.5. The aluminium hydroxide was then dissolved in alkali, heated, and the solution was treated with arsenomolybdic acid reagent. The estimations were carried out using a Leitz two-stage colorimeter. A blank and a standard solution were also run through the method. For each estimation, the colour

produced by the unknown solution was compared with that due to the adrenaline standard solution, the blank value being subtracted from each.

Addition of adrenaline to rabbit's blood, 1 µg. per ml., gave recoveries of 75 to 80 per cent of added adrenaline. Shaw found good agreement between his colorimetric method and biological assay (pressor effect) of adrenal extracts. Although the method could estimate 0.04 µg., it was preferable that the unknown solution should contain at least 0.1 to 0.5 µg. The limit of sensitivity for tissue extracts was 10^{-7}. (In human blood, Shaw found 16 to 20 µg. adrenaline per litre).

The method of Shaw was employed by Sarfy (1938, 1939) and by Tietz, Dornheggen and Goldman (1940). Bloor and Bullen (1941) encountered several difficulties with the technique. Variability in the value of the blank solution had been experienced by Whitehorn (1935) and by Shaw (1938), and Bloor and Bullen automatically corrected for the blank solution by using it as the reference in the photoelectric colorimeter. In the preliminary acid adsorption, one drop of sulphuric acid, added to the blood filtrate as suggested by Shaw, was found to be insufficient to prevent adsorption of adrenaline on aluminium hydroxide. More acid was required, which had the effect of dissolving a large part of the aluminium hydroxide. Again, in the addition of alkali suggested by Shaw to increase sensitivity, the aluminium hydroxide did not dissolve completely, and the increase in colour was small and uncertain. Bloor and Bullen therefore modified the

procedure by omitting the preliminary acid adsorption and the pretreatment with alkali.

During their early work with the method, colours corresponding to values of 0.2 to 0.5 µg/ml. adrenaline were found in the venous blood of dogs and humans. They utilised the instability of adrenaline in alkaline solution to determine whether adrenaline was in fact responsible for the blue coloration produced by a given sample. This required two estimations, with and without alkali, the amount of adrenaline present being shown by the difference between the two values (corrected for the 10 per cent by which the values for reducing substances present in blood were elevated by the alkali treatment). The colour produced by venous blood was stable to alkali under conditions in which adrenaline added to blood was completely destroyed. Blood extracts, to which adrenalinehad been added, when treated with alkali, gave the same or slightly higher values than extracts containing no adrenaline. This cast serious doubts on the existence of any adrenaline in venous blood - the limit of sensitivity of the method used by Bloor and Bullen was 0.001 µg. per ml. and they claimed an accuracy of 5 per cent in recovering 0.015 to 0.25 µg. adrenaline.

Raab (1941) considered Shaw's method to be non-specific, and initially believed that the colour corresponding to adrenaline in the blood was produced by adrenaline-steroid complexes. This conception was later abandoned, but Raab (1943) showed that the method of Shaw estimated adrenaline-like substances containing a catechol

nucleus, and ascorbic acid, which Raab found to be quantitatively adsorbed on aluminium hydroxide at pH 8.5.

Shaw's method was employed by West (1947) in a modified form using blood dialysate in the course of a comparison of colorimetric, fluorimetric, and biological tests for adrenaline in rabbit's blood. Although recovery of added adrenaline was satisfactory, "normal" values for the adrenaline content of rabbit blood, 120 µg. per litre, were excessively high. Furthermore, the use of aluminium hydroxide during the blood dialysis (Jorgensen, 1945) suggests that a proportion of the adrenaline present would be removed by adsorption, indicating that the colour estimated as adrenaline was in fact largely produced by spurious factors.

Methylene blue was employed by several workers for the estimation of adrenaline in tissue extracts (Thunberg, 1918; Ahlgren, 1921; Euler and Liljestrand, 1929); the method depends on the rate of decay of the blue colour under the influence of adrenaline. Using this method, von Euler (1933) reported a figure of 0.0001 µg. adrenaline per 100 ml. in human blood. The results were said to be affected by blood glucose (Konschegg and Monauni, 1938), and the method did not gain wide acceptance.

Before considering the fluorimetric techniques of adrenaline estimation, mention should be made of the iodine method of Euler and Hamberg (1949). This depends on the different rates of oxidation of adrenaline and noradrenaline with iodine, at pH 4 and pH 6; the colour produced by

adrenochrome and noradrenochrome (and their iodo-derivatives), at 1½ and 3 minutes after the addition of iodine, allowed the differential estimation of a mixture of the two amines. Although suitable for the estimation of adrenal and other tissue extracts when purified by adsorption of amines on alumina, the sensitivity of the method does not allow the determination of adrenaline in amounts below 10 µg. (Euler and Floding, 1955).

Fluorimetric chemical estimation of adrenaline in blood

Loew (1918) was the first to observe a yellowish green fluorescence when strong alkali was added to solutions of adrenaline. Paget (1930), during the colorimetric estimation of adrenaline with amonium molybdate reagent, observed that the reddish-brown colour disappeared and was replaced by a green fluorescence when sodium carbonate was added. The green fluorescence allowed the estimation of adrenaline 1/2,000,000. Barker et al. (1932) noted that addition of alkali to adrenaline solutions which had been treated with ferric chloride produced a yellow fluorescence similar to that produced in the ammonium molybdate test. This reaction appeared to be a very sensitive and reliable qualitative test for adrenaline.

In 1934, Gaddum and Schild described a test for adrenaline in pure solution, the essential reagents being adrenaline, oxygen, and alkali (in this case 5 N. sodium hydroxide (17)). A definite green colour was produced by adrenaline 10^{-8}. Several substances chemically related to adrenaline were tested, and the fluorescence produced twenty seconds after adding alkali was estimated as a percentage of that produced by adrenaline. The findings were:

"dopa" 3 per cent;
noradrenaline 2 per cent;
epinine 2 per cent;
catechol 0.1 per cent;
tyramine and ephedrine, nil.

In sensitivity this test represented a definite advance over the earlier chemical methods for the estimation of adrenaline. However, attempts to use the method for blood were unsuccessful. Gaddum and Schild found that the fluorescence due to low concentrations of added adrenaline was obscured by blue fluorescence due to proteins and other substances which could not be removed; also, and of considerable importance, it was found that deproteinisation by means of trichloroacetic or metaphosphoric acid removed added adrenaline. This observation was later confirmed by D'Silva (1937), who found that no more than 61 per cent of adrenaline added to plasma (0.1 µg. per ml.) was recovered when the plasma was deproteinised with trichloroacetic acid. Also, during ultrafiltration of plasma containing adrenaline (0.05 to 0.2 mgm. per ml.) there was a 17 per cent loss of adrenaline, due either to oxidation, or more probably to the combination of adrenaline with plasma proteins. The effects of deproteinisation were later emphasised by Lehmann and Michaelis (1942a) who found losses of 50 per cent of added adrenaline; von Porat (1946) who reported the loss to be considerable; and Lund (1949c) who found that removal of proteins involved a loss of 60 to 70 per cent of added adrenaline.

The fluorescence reaction described by Gaddum and Schild (1934), although used without success for blood, plasma, and serum by Tieg (1934) and by Konzett and Weiss (1939), became the basis for later methods of estimation of adrenaline in blood (Hueber, 1940; Kalaja

and Savolainen, 1941; Lehmann and Michaelis, 1942b).

The problem of separation of non-specific interfering substances from blood presented even greater difficulties with the fluorescent techniques than with the colorimetric methods because of the greatly increased sensitivity obtainable. Von Hueber (1940) suggested the use of dialysis as a means of separating adrenaline from blood (18). This was adopted and modified by Kalaja and Savolainen (1941), who used haemolysed blood, with a buffer solution (19) of pH 4 as receiving fluid to which aluminium hydroxide was added to remove adventitious fluorescing substances from the dialysate. Jorgensen (1945) employed a similar method, but used sodium citrate as an anticoagulant, which may have adversely affected the results because of displacement of pH to about 7, at which adrenaline would be largely adsorbed by the aluminium hydroxide. However, Jorgensen found an average level of 68 µg. adrenaline per litre in peripheral venous blood.

Pekkarinen (1948), using dialysis and omitting dilution of the blood with 0.01N HCl (previously used for stabilisation of adrenaline), displaced the pH of the dialysate to about pH 8.5, at which adrenaline was largely adsorbed. The precipitate was then dissolved in sodium hydroxide and the fluorescence of the solution measured. Pekkarinen found no adsorption of interfering substances which, it should be remembered, was the original object of using aluminium hydroxide in the dialysis method. The sensitivity of the method as used

by Pekkarinen was 5 to 10 µg. per litre; no adrenaline was found in peripheral blood under normal conditions, although he estimated a value of 10 to 100 µg. per litre in the adrenal vein of dogs under ether anaesthesia.

Von Porat (1946) found the dialysis method to be unserviceable because of an inhibitory effect on adrenaline fluorescence produced by the blood dialysate; it was later shown (Bloch, 1948) that addition of a blood dialysate to an aqueous solution of adrenaline was able to almost totally inhibit the development of fluorescence on addition of strong alkali. Also, von Porat found that the dialysis method gave high blank values and uncertain quantitative recovery.

Lehmann and Michaelis (1942a) had earlier criticised the dialysis method on the ground that there was a loss of adrenaline in the process of dialysis; that there was no control over oxygen tension during dialysis; and that the presence of heavy metal ions, especially iron, inhibited the development of fluorescence - this was investigated by Jorgensen (1945), who found no interfering effect from heavy metal ions present in normal blood dialysates.

Lehmann and Michaelis (1942b) employed small quantities of plasma, and after addition of alkali the fluorescence was estimated in a photofluorimeter; they attempted to eliminate the blue fluorescence produced by proteins by means of suitable interference filters.

Von Porat (1946) modified the method of Lehmann and Michaelis by achieving protein precipitation with

tungstic acid, which strongly activated the adrenaline fluorescence. By this means, it was possible to demonstrate 0.25 µg. adrenaline per ml. in recovery experiments, but no adrenaline could be demonstrated in plasma.

West (1947) using a dialysis method followed by fluorimetric estimation (based on the method of Jorgensen, 1945) estimated the average adrenaline content of rabbit blood to be 100 µg. per litre.

Annersten et al. (1949) pointed out that the use of blood plasma (Lehmann and Michaelis, 1942b) had given notably higher adrenaline values than the blood dialysate method (von Hueber, 1940; Kalaja and Savolainen, 1941; Jorgensen, 1945; West, 1947). Annersten et al., carried out separate determinations on blood and plasma dialysates and impermeates, and on plasma. While direct estimation on blood plasma, and on the plasma impermeate, yielded figures of 1 to 3 µg. adrenaline per ml., 0.07 µg. per ml. was found in the dialysates, showing that only a small percentage of the adrenaline had passed through the collodion membrane. Their conclusion was that the two modifications of the fluorescent method yielded incompatible results. On the one hand, free dialysable adrenaline was determined, whereas on the other, total adrenaline content including free and protein-bound adrenaline. However, in view of the excessively high values found in plasma and in plasma dialysates by Annersten et al., their conclusions must be seriously in doubt.

It must be admitted that the early fluorimetric methods for the estimation of adrenaline in blood were a

considerable advance over colorimetric techniques both in sensitivity and specificity. However, there were many problems remaining. One of these was the unsuitability of the dialysis method for the extraction of adrenaline from blood, because of poor recovery of adrenaline, an inhibiting effect on adrenaline fluorescence, and the inadequate separation of interfering substances. Disputes on the suitability of the dialysis method were also due in part to the lack of knowledge of the influence of acidity on the adsorption of adrenaline by aluminium hydroxide.

The use of whole plasma was certainly no more satisfactory, because of the introduction of adventitious fluorescence, which was not overcome by the use of interference filters during measurement. The fluorescence of the sample was compared with eosin standard solutions (Kalaja and Savolainen, 1941; Pekkarinen, 1948), or it was measured by Pulfrich's photometer (Lehmann and Michaelis, 1942b). Von Porat (1946) used a glass fluorescence standard. The extremely weak fluorescence shown by the small quantities of adrenaline present in blood dialysates, and the spurious fluorescence introduced by contaminating substances which were incompletely separated from the solutions whose fluorescence was being measured, must have made for great inaccuracy with these available methods of measurement. Also, when standard fluorescent solutions were used for comparison with the sample, they were calibrated against pure solutions of

adrenaline. This left out of account the possibility that the fluorescence due to adrenaline was inhibited by substances present in blood dialysates.

An attempt has been made to bring to the fore the salient features of the earlier chemical methods utilised for the estimation of adrenaline in blood, prior to the recent techniques of Lund (1950), and Weil-Malherbe and Bone (1952a). A table adapted from Pekkarinen (1948), which summarises the results obtained with all the reported methods prior to 1949, is provided on pages 98 - 100.

The Oxidation of Adrenaline

Following the observations by Vulpian in 1856, and in the course of the early attempts to estimate adrenaline by agents such as ferric chloride, potassium iodate, mercuric chloride, and potassium persulphate, it became appreciated that the colour reactions were brought about by the oxidation of adrenaline, usually to a red coloured oxidation product. The chemical nature of the oxidation product was for many years unknown. In 1933, Ball and Chen measured the oxidation products of the systems formed by treating adrenaline with oxidising agents, and obtained evidence that the primary product formed was generally the corresponding ortho-quinone (page 102). This was extremely unstable, and at pH 7 was found to change within 1/30 second, irreversibly, to give a product of undetermined chemical structure. In 1937, Green and Richter isolated this compound, which contained four hydrogen atoms less than adrenaline, and it was shown to have the properties of N-methyl-2:3-dihydro-3-hydroxyindole-5:6 quinone. This substance is a condensation product between carbon atom 2 in the benzene nucleus of adrenaline and the amino-group under the ring formation. It was named "adrenochrome" by Green and Richter.

Adrenochrome was found to be very unstable, and in solution even under the optimum conditions of storage, at pH 4 and a temperature of 0°C, there was considerable decomposition and melanin formation in four to five hours (20).

The Production of Fluorescence

It was shown by Gaddum and Schild (1934) that oxygen was required for the production of fluorescence when alkali was added to a solution of adrenaline. Shaw (1941) ascribed the fluorescence to adrenaline quinone, while Rangier (1945) suggested the isomeric leuco-adrenaline (21). Ehrlen (1948) was the first to identify the fluorescent compound as 1-methyl-3:5:6:-trihydroxyindole (page 102). This was independently verified by Fischer (1949), by Lund (1949b), and by BuLock and Harley-Mason (1951).

Lund (1949a) showed that addition of alkali to oxygen-free solutions of adrenochrome produced a strong fluorescence almost at once, the fluorescence being reduced by addition of oxygen; thus the fluorescent substance was at the same degree of oxidation from adrenaline as was adrenochrome. Oxidation of adrenaline in acid solution gave the red non-fluorescent adrenochrome, while oxidation in alkaline solution formed the yellowish-green fluorescent substance. Lund (1949b) prepared the fluorescent compound in crystalline form, by rearrangement of adrenochrome in oxygen-free solution in the presence of a strong base, subsequently precipitating the fluorescent substance with acid. He named it "adrenolutine" and suggested the sequence of events shown on page 102 for the oxidative breakdown of adrenaline.

Following his identification of adrenolutine, Lund (1949c) introduced his method for the estimation of adrenaline in blood. Although in many respects new, Lund's

method was dependent on the production of fluorescence with alkali, as previously described by Gaddum and Schild (1934). As in every method for the chemical estimation of adrenaline in blood, two distinct problems were involved - the separation of adrenaline from blood, and the estimation of the separated adrenaline.

(1) The isolation of adrenaline from blood

In face of the confusion arising from the use of aluminium hydroxide for adsorption of adrenaline and other substances at different pH values, Lund investigated the effect of pH on the adsorption of adrenaline by aluminium hydroxide and aluminium oxide. With aluminium hydroxide no adsorption of adrenaline occurred between pH 4 and 5. With rising pH values, increasing amounts of adrenaline were adsorbed, but at pH 8 to 9 adsorption was still incomplete. With aluminium oxide (Al_2O_3 for chromatography, B.D.H.) adsorption and acidity were similarly interrelated, but 94 per cent adsorption occurred at pH 8.4 (page 101). By using aluminium oxide contained in a glass column through which the samples were drawn by suction, 92 per cent of adrenaline was recovered from pure solution. Loss of adrenaline on the column was considered to be probably due to oxidation in the alkaline solution during filtration. This implied that the necessity to keep the alkalinity sufficiently low to prevent oxidation of adrenaline conflicted with the need to keep the pH of the adrenaline solution at about 8.4, where adsorption of adrenaline was at a maximum.

From plasma samples, 80 per cent recovery of added adrenaline (20-200 µg. per litre) was consistently obtained. Lund attributed the 20 per cent loss with plasma filtrates to washing of the column with sodium acetate.

2. The estimation of adrenaline

Lund's method was based on the quantitative oxidation of adrenaline to adrenochrome, this reaction being complete after a plasma eluate was shaken with manganese dioxide for 30 seconds; the adrenochrome was then further oxidised with 20 per cent sodium hydroxide to the fluorescent compound adrenolutine. When alkali had been added, further oxidation of adrenolutine was prevented by means of ascorbic acid (Ehrlen, 1948), the affinity of sodium ascorbate for oxygen being considerably greater than that of adrenolutine.

Lund affirmed that the use of manganese dioxide resulted in a considerable increase in sensitivity; the maximum fluorescence after addition of alkali to adrenaline solutions was only one-fourth to one-third of that measured when the adrenaline solution had been previously oxidised with manganese dioxide.

In 1950, Lund modified his original procedure to allow the simultaneous estimation of adrenaline and noradrenaline in the same sample. Noradrenaline was

found to behave like adrenaline with regard to adsorption on aluminium oxide, quantitative adsorption occurring at pH 8.5. The oxidative process, noradrenaline ⟶ noradrenochrome ⟶ noradrenolutine was analogous to that for adrenaline. However, it was found that while adrenaline was quantitatively oxidised to adrenochrome within the pH range 3 to 7, only 5 per cent of noradrenaline was oxidised to noradrenochrome at pH 3, although quantitative oxidation occurred at pH 6.5. This was utilised for the differential estimation of adrenaline and noradrenaline in blood. Because of the importance of Lund's technique and its excellent specificity, the technique of estimation will be briefly described.

After chromatographic adsorption of plasma adrenaline and noradrenaline on a column of aluminium oxide (this is detailed in the subsequent description of the ethylene diamine condensation method employed by the author), elution is carried out with 10 ml. 0.2N acetic acid and 10 ml. water. The eluate is divided into three portions, each of 10 ml. A, B, C.

(A) This is the blank solution; with it, by potentiometric titration, is estimated the required amount of 0.8M sodium acetate or 0.2N hydrochloric acid to bring solutions of C and B to pH 6.5 and 3.0 respectively. A is then shaken for 20 seconds with 0.1 gm. manganese dioxide (previously washed with acetic acid and water to get rid of fluorescent substances) at pH 6.5, centrifuged

for 30 seconds at 3000 rpm and filtered (the filter paper having been washed free of fluorescent substances). To 8 ml. of the filtrate is added 0.84 ml. of 20 per cent sodium hydroxide. Oxidation of adrenaline to adrenochrome and rearrangement to adrenolutine then occurs, followed by complete oxidation of adrenolutine within 2 to 3 minutes. Five minutes after adding the alkali, 0.16 ml. of 1 per cent ascorbic acid is added to the faded blank solution.

(B) Is used for the determination of adrenaline. The previously determined amount of 0.2N hydrochloric acid is added to bring the pH to 3.0. The solution is then oxidised for 60 seconds with manganese dioxide. After centrifuging and filtering, 1 ml. of a mixture of 1 ml. 20 per cent sodium hydroxide and 0.20 ml. of 1 per cent ascorbic acid is added to 8 ml. of the filtrate. As in solution A, the adrenaline is oxidised to adrenochrome and rearranged to adrenolutine, but further oxidation is prevented by the ascorbic acid.

(C) Is used for determination of adrenaline plus noradrenaline. The previously determined amount of 0.8M sodium phosphate is added to the sample, bringing the pH to 6.5. The solution is oxidised for 20 seconds with manganese dioxide. After centrifuging and filtering, 1 ml. of the sodium hydroxide-ascorbic acid mixture is added to 8 ml. of the filtrate.

The fluorescence of solutions B and C could be

measured immediately after the addition of sodium hydroxide-ascorbic acid, but it was necessary for the blank solution to stand for about 30 minutes to allow its fluorescence to fade completely. It was previously mentioned that at pH 3.0 only about 5 per cent of the noradrenaline present was oxidised to noradrenochrome. Moreover, the fluorescence shown by noradrenolutine was just over half of that produced by adrenolutine, so that the flouresscence due to noradrenaline in solution B was usually only a small fraction of that due to adrenaline. Therefore, to simplify the calculation of results, Lund ignored the fluorescence derived from noradrenaline in solution B.

A sensitive photofluorimeter is necessary to record the fluorescence intensity of the three solutions.

For calculation of results, Lund employed previously determined calibration curves (galvanometer deflections plotted against fluorescence intensity). The deflections produced by solution A were subtracted from those given by solution B, and from the adrenaline calibration curve the adrenaline concentration was found. The deflections produced by solution B were then subtracted from those produced by solution C, and from the noradrenaline calibration curve the noradrenaline concentration in the eluate was determined. Previous correction was made for dilution of solutions A and B with hydrochloric acid and sodium phosphate solutions, and the concentrations determined were corrected by the factor 1.25, because of the constant 20 per cent loss of amines, thought to be due to washing

of the column with sodium acetate.

The adrenaline concentration in plasma could be estimated with an accuracy of ± 5 per cent with concentrations above 10 µg. per litre and ± 10 per cent within the range from 1 to 10 µg. per litre. For noradrenaline the lower limit of sensitivity was 10 µg. per litre, the accuracy rarely exceeding 10 per cent if the concentrations of adrenaline and noradrenaline were of the same order.

Specificity of the Fluorescence Reaction

Lund investigated several compounds chemically related to adrenaline (page 103).

Propynaline and noradrenaline proved to be the only two of these compounds which on oxidation and addition of alkali yielded the yellowish-green fluorescence characteristic of adrenaline.

The requirements for this fluorescence reaction are:

(1) Indole ring closure - determined by the presence of a secondary amino group.

(2) Formation of the quinone - two adjacent OH groups are required on the benzene ring.

(3) Autoreduction to give the fluorescent oxidation product requires, on the side chain, a hydroxyl group at the β -carbon (next to the benzene ring), and hydrogen at the α -carbon (Lund, 1949c; Bu'Lock and Harley Mason, 1951).

As Lund stated, "the only two remaining possibilities for substitution are therefore at the α and β carbon atoms of the side chain. Such substitution products are unknown, either as drugs or as intermediary substances normally present in the organism. Numerous other types of compounds show yellowish-green fluorescences, e.g. indoxyl derivatives (22), of which a few occur as metabolic waste products in the organism. However, indoxyl propionic acid and 2-carboxy-indoxyl-acetic acid proved to be non-fluorescent as were related compounds such as the amino-acids tyrosine and tryptophan."

Although not tested by Lund, isopropylnoradrenaline, in accordance with its chemical structure, gives a strong fluorescence with this method (Von Euler and Floding, 1955); while small amounts of isopropylnoradrenaline have been tentatively identified in adrenal extracts (Lockett, 1954), their presence under normal physiological conditions is not established. A very weak fluorescence is also produced by dopamine and 3:4-dihydroxynorephedrine (Corbasil, cobefrine) (Von Euler and Floding, 1955), (23).

Weil-Malherbe and Bone (1952a) criticised the method of Lund for these reasons:

(1) Recovery of added adrenaline was only 80 per cent.
(2) The sensitivity for adrenaline was above 10 µg. per litre of blood.
(3) It was necessary to remove manganese dioxide rapidly and completely.

(4) The possibility existed that adrenochrome might be reduced by ascorbic acid, thus escaping rearrangement to adrenolutine in alkaline solution.

(5) Variations in temperature, and in the manganese dioxide employed, might interfere with the method.

Although inadequate sensitivity was an important drawback to the application of Lund's method, this could be improved by the use of a more sensitive photofluorimeter (Weil-Malherbe and Bone,1952a; Valk, 1955; Ludemann et al. 1955). By incorporating several modifications into the method of Lund, including the use of a Farrand photofluorimeter, Ludemann et al. (1955) increased reproducibility and sensitivity, allowing the estimation of 2 µg. adrenaline per litre of plasma. Oxidation of the standard solutions at the same pH as the plasma eluates (pH 3.5 to 4.1) apparently overcame the constant loss of 20 per cent incurred by Lund (1949c); 102 per cent recovery of added adrenaline (10 µg. per litre) was achieved. However, sensitivity of the method remained inadequate; control levels of adrenaline concentration in 5 human subjects and 7 dogs were 0 to 4 µg. per litre of peripheral arterial blood, while it was apparently not possible to estimate levels between zero and 1 µg. per litre, which appears to be the true normal range. Furthermore, extremely high concentrations - 30 to 480 µg. adrenaline per litre - were estimated during severe anoxia in dogs.

A further modification of Lund's method has recently been described by Von Euler and Floding (1955). Potassium ferricyanide (0.25 per cent) is employed in preference to manganese dioxide for the oxidation of adrenaline and noradrenaline, and zinc sulphate (0.5 per cent) is added to increase the speed of formation of adrenochrome at pH 3.5. In other respects, the method is closely similar to the technique described by Lund except for the use of a more sensitive fluorimeter designed after the principles given by Weil-Malherbe and Bone (1953). According to Von Euler and Floding, "the method is rapid and easy to perform in any laboratory having access to a suitable fluorimeter."

Although utilised by these workers for the chemical assay of adrenaline in urinary extracts, the technique appears to be adaptable to the estimation of plasma concentrations of adrenaline and noradrenaline.

The method of estimation used in the studies to be described in this thesis is essentially similar in technique to that described by Weil-Malherbe and Bone (1952a, 1953). The basis of the method is a condensation reaction of catechol amines with ethylene diamine (24),(p.104), originally described by Natelson *et al.* (1949). Natelson pointed out that the fluorescence produced by the oxidation of adrenaline in alkaline solution, excited by a wavelength of 365 mμ, was not specific for adrenaline; various substances such as protein hydrolysates, aged

tryptophan, and tyrosine, produced fluorescence under similar conditions (25). Also, the fluorescence was transient, a fact responsible for considerable difficulty in measurement, as previous workers had found. Again, the fluorescent material was not completely extractable with organic solvents, the fluorescence being affected by salt concentration. Natelson found that treatment of adrenaline solutions with an organic primary amine such as ethylene diamine, butylamine, propylenediamine, benzylamine, aniline or O-phenylenediamine, produced a fluorescence excitable by a wavelength of about 435 mμ, which did not excite the interfering substances listed above.

The fluorescent substance was found to be extractable into aliphatic alcohols, e.g. butyl or amyl alcohol. Fluorescence was quite stable and remained constant for several days. The maximum emission of fluorescent light occurred at about 570 mμ, there being negligible emission below 500 mμ.

This reaction was not further studied by Natelson et al., but Weil-Malherbe and Bone (1952a) adopted it as the basis of their method for the estimation of adrenaline in blood. They pointed out that oxygen was necessary for the condensation reaction, and that if adrenaline was replaced by adrenochrome a fluorescence intensity curve was obtained, both in the presence and absence of oxygen, which was identical to that produced by equimolar

amounts of adrenaline in the presence of oxygen.

In the method first described, the adrenergic amines were collectively estimated as "adrenaline-like substances." The fluorescence intensity produced by noradrenaline was one fifth that produced by equimolar amounts of adrenaline. The fluorescence produced by a mixture of both substances was equal to the sum of the fluorescences produced by the component parts. In a subsequent paper, Weil-Malherbe and Bone (1953) modified the method to allow the differential estimation of adrenaline and noradrenaline in plasma samples. The use of two separate interference filters, and the increase in the sensitivity of the fluorimeter, allowed advantage to be taken of the maximum fluorescence emission of adrenaline at about 580 mμ, and for noradrenaline at about 450 mμ.

The initial separation of catechol amines from plasma samples was accomplished, as in the method of Lund (1949c), by adsorption on a column of aluminium oxide.

Weil-Malherbe and Bone cited these advantages for the ethylene diamine method:

(1) The unstable, highly reactive adrenochrome was trapped in the nascent state and forthwith converted into a completely stable condensation product.

(2) The fluorescence remained constant for at least 24 hours.

(3) A sensitivity of 1 μg. per litre of blood could be obtained.

(4) Recovery was quantitative; no correction factor was needed.

(5) Additive results were obtained with adrenaline and noradrenaline.

Specificity of the method

Weil-Malherbe and Bone found that fluorescence of variable intensity was produced by all the catechol derivatives tested, with the exception of dihydroxyphenylalanine. They alleged that this finding was of little practical importance since physiological catechol derivatives such as dihydroxyphenylalanine were largely eliminated by the extraction procedure,(pages 105 - 106).

Direct extraction of plasma into isobutanol (omitting the addition of ethylene diamine) showed that fluorescence was less than that of the usual reagent blank. This indicated that none of the fluorescence was due to preformed plasma components, but was all produced by condensation with ethylene diamine.

Other experiments performed by Weil-Malherbe and Bone appeared to show that:

(1) Added adrenaline disappeared from plasma at about the same rate as the preformed estimated adrenaline, when auto-oxidised by shaking in air.

(2) Extract of rabbit liver removed preformed material and added adrenaline from plasma at an equal rate; the liver extract was inactivated by boiling and inhibited by ephedrine, a specific inhibitor of amine oxidase. It

was concluded that the liver extract owed its activity to the presence of amine oxidase. This reaction was further shown to be quantitative, the plot being that of a first order reaction, indicating that the reacting material was either a single substance or a mixture of substances with a similar affinity for the enzyme.

Paper chromatographic experiments were carried out by Weil-Malherbe and Bone to obtain evidence of specificity. N-butanol was used to extract the plasma catechols from 150 ml. blood. The extracted amines were then re-extracted with 0.05N HCl and the neutral concentrated solution passed over a column of aluminium oxide. The amines were eluted with acetic acid and the eluate evaporated. The residue was transferred to paper with acid acetone and chromatographed by the method of Goldenberg et al. (1949). Sections of the chromatogram were extracted with dilute HCl and the adrenergic amines in the extract were estimated fluorimetrically.

The fluorigenic material in plasma was stated to be concentrated in positions exactly opposite those of the adrenaline and noradrenaline spots on a reference strip. By employing the method developed for differential estimation of the two amines (Weil-Malherbe and Bone,1953), it was found that the substances with the same Rf value as noradrenaline and adrenaline also yielded on condensation with ethylene diamine a fluorescence with the same spectral properties as that produced by condensation of noradrenaline and adrenaline respectively (26).

Recovery

Both adrenaline and noradrenaline were stated to be quantitatively recovered when pure solutions were estimmated. Additions of adrenaline to horse serum, in amounts equivalent to 2, 3, 5 and 10 µg. per litre, resulted in a quantitative recovery with a standard deviation of 5 per cent over this range. The values quoted for the differential method were - adrenaline, 96.4 per cent ± 5.44 per cent, at levels of 2,3,4,5,10 and 15 µg. per litre; noradrenaline, 100.7 per cent ± 6.79 per cent, at levels of 5,10,15 and 20 µg. per litre. This evidence of quantitative recovery of added amines up to 100 per cent is in disagreement with the 80 per cent recovery quoted by Lund (1949c).

Adrenaline, 1 to 1.5 µg., and noradrenaline, 5.2 µg. per litre of peripheral venous blood were reported by Weil-Malherbe and Bone (1953) to be average normal concentrations in healthy human subjects. However, Weil-Malherbe reported in 1955, in a personal communication to the author, that normal values for blood adrenaline and noradrenaline concentrations as estimated in his laboratory were now found to be somewhat lower than those figures published in 1953. He attributed this to modifications to the photofluorimeter and to (unspecified) changes in the anticoagulant solutions employed.

Manger et al. (1953) described the use of sodium thiosulphate for its differential effect on the fluorescence of adrenaline and noradrenaline, enabling separate

estimation of the two amines by the method of Weil-Malherbe and Bone (1952a). Estimated adrenaline concentrations in peripheral plasma ranged from negative values (designated "zero" by these workers) to 0.6 µg. per litre, with a mean value of 0.14 (S.D. ± 0.21).

The use of the ethylene diamine condensation method was also reported by Aronow and Howard (1955) for the assay of adrenal venous blood; the technique was basically similar to that of Weil-Malherbe and Bone (1953), but the use of adsorption columns was discarded, alumina being added directly to plasma extracts. A limited experience with this technique did not demonstrate to the author that it held any advantage over the method described in this thesis which is based on column adsorption.

A description of the ethylene diamine condensation method, with the modifications employed by the author for the studies on insulin hypoglycemia, now follows:

Reagents

(1) Alumina. Wöelm alumina (aluminium oxide), non-alkaline, Grade 1 (Alupharm Chemical Co., Elmont, Long Island) was used without further treatment, and could be stored without desiccation.

(2) Glass-distilled water. Enough glass distilled water was prepared for one week's use to avoid contamination, and was kept at room temperature in glass stoppered containers. This water was used throughout the procedure and in making up all reagents, except where

otherwise stated.

(3) Ethylenediamine mixture. Ethylenediamine .2HCl was prepared by adding 1 part of freshly redistilled ethylenediamine base to 4 parts of absolute ethyl alcohol, followed by addition of this mixture to 30 parts of absolute ethyl alcohol containing 10 per cent concentrated hydrochloric acid. Fifty ml. of base yielded approximately 115 gm. of the dihydrochloride salt by this procedure. The precipitated ethylene diamine .2HCl crystals were then filtered on a Buchner funnel, washed with small amounts of absolute ethyl alcohol, dried in a vacuum desiccator overnight, and used to prepare a 2M aqueous solution as needed. Five parts of the freshly prepared 2M solution of ethylenediamine 2HCl and 7 parts of the freshly redistilled ethylenediamine base were chilled separately to 4°C, and then carefully mixed so as to prevent the temperature from rising above 20°C. The mixture was stored at 4°C., and quantities sufficient for immediate use were removed as needed. This prevented the gradual increase in fluorescence of blanks and the decrease in fluorescence of the adrenaline and noradrenaline standards, which occurred when the mixture was stored at room temperature. The use of 1 ml. of this mixture produced no change in fluorescence from that obtained with 1.2 ml. as called for in the original method described by Weil-Malherbe and Bone (1952a).

(4) Sodium acetate. A 2M solution of Merck reagent sodium acetate was processed through a column of "Zeo-Rex" to remove contaminating heavy metals (Zeo-Karb" 215, as used by Weil-Malherbe and Bone, is unobtainable in America). Two gm. of "Zeo-Rex" were soaked in water overnight, layered onto small gravel in a chromatograph column, and washed with 3 litres of 2N hydrochloric acid, followed by 3 litres of water and 6 litres of 4 per cent sodium chloride. Excess sodium chloride was then removed with 1 litre of water and the acetate solution passed through at a rate of about 10 ml. per minute. The solution was stored in a glass-stoppered bottle, and diluted 1:10 with water as needed.

(5) Acetic acid. Enough 0.3M acetic acid was made up to last for approximately one week, and was stored at room temperature.

(6) Sodium thiosulphate-sodium fluoride mixture. A solution containing 30 gm. of sodium thiosulphate (reagent grade) and 20 gm. of sodium fluoride (reagent grade) in 1 litre, stored at room temperature.

(7) Sodium chloride. Any reagent grade sodium chloride was found satisfactory.

(8) Isobutyl alcohol. Merck reagent isobutanol, labelled "suitable for fluorimetric analysis" was used without further treatment.

(9) Stock solutions of adrenaline and noradrenaline. Solutions of adrenaline bitartrate and noradrenaline bitartrate monohydrate in 0.1M acetic acid were

prepared by dissolving 36.3 and 39.6 mg. respectively of the salt in 100 ml. of 0.1M acetic acid, with further dilution 1:5000 to obtain solutions which contained 40 µg. of base per litre. When stored at refrigerator temperatures, these solutions remained stable for at least six months as determined by assay with this method.

(10) Permanent standards. Ten ml. isobutyl alcohol extract of 10 ml. of water containing 20 µg. adrenaline which had been condensed with ethylene diamine; this was stored at 4°C. and diluted 1:100 with isobutyl alcohol for use as required to maintain instrument sensitivity at a preassigned value (90.0 divisions at diaphragm opening No. 3) for each secondary filter.

Permanent standards of adrenaline condensate in isobutyl alcohol were stable for at least six months when stored at 4°C. Using one dilution of standard, the readings of blank solution with the yellow filter pair in position were about 30 per cent of those obtained with the green filter pair. Since the estimated adrenaline levels in normal subjects were found to be about 4 per cent of apparent noradrenaline levels, the use of a larger galvanometer deflection with the yellow filter pair appeared justifiable. However, two dilutions of the standard could be used to achieve comparable precision of the galvanometer reading with both secondary filter pairs.

Apparatus

(1) Chromatographic columns. These were made for the author and are of simple design. A cylindrical bulb

of about 30 ml. capacity is joined at one end to a 10 cm. length of 7 mm. tubing which has a small constriction at the opposite end; the other end of the bulb is joined to a 5 cm. length of 10 mm. tubing. This tube receives a rubber hose for applications of air pressure during chromatography, and all solutions are introduced at this end of the column.

(2) Fluorimeter. A Farrand fluorimeter, with a Rubicon galvanometer to receive the output of the photo-multiplier valve circuit, was found to have adequate sensitivity for the measurement of the low level of fluorescence intensity encountered (27). The primary filter combination consisted of an interference filter with a peak wavelength of 436 mμ plus a Wratten gelatin filter No. 35. The secondary filter combinations were:

(a) Yellow filter pair, comprising two Wratten gelatin filters Nos. 57 and 21, with a combined peak at approximately 580 mu.

(b) Green filter pair, comprising a 500 mμ interference filter plus a Wratten gelatin filter No. 57.

Method

Before investigating the effect of insulin hypo-glycemia on the adrenaline concentration in peripheral blood, the author, while learning the technical procedure, assisted in a preliminary assessment of the method of Weil-Malherbe and Bone (1953). Some of the results of this evaluation will be included here.

The estimation of blood samples was carried out in duplicate for all the studies described in this thesis. For this purpose, approximately 32 ml. of blood was withdrawn into a 30 ml. syringe previously moistened thoroughly with heparin, 1000 units per ml. (It was previously established that heparin did not interfere with the analysis). The blood was transferred to a chilled centrifuge tube, which was centrifuged at about 300 G. for 20 minutes. The plasma was aspirated, and divided into two samples, each of 8 to 10 ml., which were then diluted with 5 ml. of fluoride-thiosulphate solution. For this study, the diluted plasma was stored overnight in the refrigerator at approximately 4°C.

Adsorption columns were prepared by pouring 0.7 gm. of alumina into a column partly filled with distilled water, allowing the alumina to settle by gravity onto a glasswool plug previously placed at the constriction. As the alumina settled, the glasswool was gently tapped with a glass rod. Five ml. of 0.2M sodium acetate were then passed through the column until about three quarters of its volume was filtered.

The diluted plasma was now further diluted with an equal volume of 0.2M sodium acetate solution (adjusted to pH 8.4). Adjustment of the plasma-acetate mixture to pH 8.4 was not found to be critical. Additions of adrenaline and noradrenaline to plasma, with and without pH adjustment, were made in quadruplicate and recovered in

equal amounts; alumina in sufficient quantity acts as an ion-exchange adsorbant and 0.7 gm. in a column will raise the pH of the effluant by about one pH unit provided 25 per cent of the fluid is plasma (Valk, 1955).

The plasma-acetate mixture was added to the column and passed through at a rate of about 30 drops per minute. Before all the plasma had been filtered, 5 ml. 0.2M sodium acetate were added. This was followed by 5 ml. of water, which was forced through until the last few drops just entered the alumina column.

Elution of the adsorbed amines was effected by passing 5 ml. of 0.3M acetic acid followed by 5 ml. of water through the column. The eluate was collected in a 50 ml. centrifuge tube, in which all the subsequent reactions occurred.

In addition to plasma eluates, two adrenaline and two noradrenaline standards, and two reagent blanks were run through the condensation procedure. It was found that when adrenaline standards were prepared for condensation in 5 ml. of 0.3M acetic acid and 4 ml. of water, fluorescence intensity was only 60 per cent of that obtained when these standards were prepared in 9 ml. "reagent blank solution." ("Reagent blank solution" - the acetic acid/water eluate obtained from a column through which reagents or water alone had been passed). The fluorescence intensity of noradrenaline standards did not differ under the two conditions of preparation. The

increase in fluorescence of adrenaline condensate was apparently due to the presence in the eluate of particles of alumina, and was not due to the reagents used. Use of water eluates from the alumina column did not increase the fluorescence of adrenaline condensates, whereas acid eluates were effective. The mechanism of this catalysis by alumina was not further studied. In the course of studies carried out in this laboratory it was found also that the fluorescence readings for "reagent blank solution" when water alone was passed through the column were well within the variation shown by reagent blank solutions obtained from a column washed with fluoride-thiosulphate, water and sodium acetate solution (Valk, 1955).

In these studies, therefore, "reagent blank solution" was prepared by making up the alumina column in the usual manner and passing through 30 ml. of water. The column was then eluted. Six reagent blank solutions were used, two being condensed without addition, the fluorescence being read as "reagent blank." The other four reagent blank solutions were used for two adrenaline and two noradrenaline standards. Standards were used which were only one-fifth the concentration of those recommended by Weil-Malherbe and Bone, 1952a,1953). They were prepared by adding 1 ml. of "stock solution of amines," containing 40 µg. per litre, to 9 ml. of the reagent blank eluate. The standards were read in the fluorimeter at the same sensitivity setting as that used for samples from normal plasma.

The condensation reaction was carried out by adding 1 ml. of ethylene diamine mixture to each centrifuge tube containing eluate. The tubes were then heated at 50°C. for 20 minutes. Four gm. of sodium chloride were now added to each tube, followed by 6 ml. of isobutyl alcohol. The tubes were shaken mechanically for 4 minutes and briefly centrifuged to clear the isobutyl layer.

All the tubes received identical time and intensity of exposure to roomlight or daylight during and after the condensation. The condensates of adrenaline and noradrenaline, and of the reagent blank solutions, were found to be sensitive to light of wavelengths less than about 500 mμ. and the fluorescence intensity of all three decreased on prolonged exposure to either the primary source of the fluorimeter or the unfiltered Hg-vapour bulb. Fluorescence decay was measured for all three condensates on a Brush pen recorder which received the output at peak gain of the photomultiplier tube via a direct current amplifier. Decay was almost completely confined to that portion of the spectrum passed by the green (500 mμ.) filter pair. The noradrenaline condensate decayed exponentially with time, and although the decay was complete and irreversible after prolonged exposure, a partial recovery always ensued if the exposure was of short

duration. Exposure of adrenaline and of reagent blank condensate for 15 minutes resulted in about 5 and 50 per cent irreversible decay, respectively, but a 10 second exposure produced no measurable decay for these condensates. Exposure to roomlight, comprising six 40-Watt fluorescent bulbs in reflectors at a distance of four feet, produced no measurable decrease in fluorescence of the noradrenaline condensate. No increase in fluorescence of the noradrenaline condensate was obtained by carrying out the condensation and all subsequent manoeuvres in red light (Valk, Price and Millar, 1955).

After centrifugation, the tubes were placed in the dark and assayed fluorimetrically by withdrawing one tube at a time and transferring 1.5 to 2 ml. of the isobutyl layer to a cuvette, which was used for all samples. The cuvette was rinsed with small amounts of isobutyl alcohol from each successive sample before transferring the final quantity of the sample to be read.

With the secondary green filter pair in place in the fluorimeter and the diaphragm aperture No. 3 in the primary light path, the galvanometer was set at 90.0 divisions with the permanent standard which had been diluted 1:50. All samples were first read with the green (500 mμ.) filter pair in position, to ensure

constancy of decay of all the noradrenaline condensates. With the shutter open, the sample cuvette was quickly introduced into the light path and the new deflection noted after an interval of 5 seconds, which was kept constant for all samples. The same procedure was followed with the secondary yellow (580 mμ.) filter pair in position. With this filter, due to the absence of fluorescence decay, the time interval prior to reading did not require to be constant, provided that time was allowed for completion of the galvanometer swing.

Calculations of plasma concentrations of adrenaline and noradrenaline were made by constructing simultaneous equations involving galvanometer divisions per ug. of adrenaline and noradrenaline per litre, for both secondary filter combinations:

Where a = average of galvanometer deflections due to adrenaline standards.

b = average of galvanometer deflections due to noradrenaline standards.

c = average of galvanometer deflections due to reagent blank.

d = galvanometer deflection due to sample.

$$\text{then } A \frac{(a - c)}{4} + N \frac{b - c}{4} = d - c$$

Two of the above equations were constructed, one for each secondary filter; they were then solved to give estimated adrenaline and noradrenaline concentration per litre in the plasma sample. If less than 10 ml. of plasma were used for estimation, the figures

obtained were multiplied by 10/volume of the sample, to give the corrected values in µg. per litre.

Additions of adrenaline, 0.001 to 0.20 µg., and of noradrenaline, 0.005 to 4.0 µg., were made to 10 ml. samples of human plasma, a total of 86 additions being made. Average recovery of added adrenaline was 78 per cent (S.D. ± 26 per cent and of noradrenaline 80 per cent (S.D.± 27 per cent).

Recoveries and standard deviations at, and slightly above the levels of adrenaline and noradrenaline found in plasma of normal human subjects, are shown on page 107. The increased standard deviation of recovery at lower levels of added adrenaline and noradrenaline is not unexpected since the variation in reagent blanks induced greater scatter as the level of added amines approached zero.

On page 108 are listed the dihydroxyphenol compounds which were added to plasma and tested by the ethylene diamine condensation method in this laboratory. Percentage of recovery, and the fluorescence ratio shown by each compound on the secondary filter are also illustrated. (This comparison was carried out by Dr. A. deT. Valk, to whom the author is grateful for permission to include here). It is of interest to note that 5-hydroxytryptamine (serotonin, enterotonin, thrombocytin) is apparently not adsorbed on aluminium oxide. The fluorescence ratio of 3:4 dihydroxyphenyl acetic acid can be seen to differ from that shown by noradrenaline by 8.0 per

cent. The possible implication of this will be more fully considered later.

Plasma levels in normal human subjects

The plasma of twenty-two normal adults was analysed for adrenaline and noradrenaline in this laboratory, using duplicate samples. The mean of the estimated adrenaline levels was 0.09 µg. per litre of plasma (S.D. ± 0.12) with extremes of -0.14 and 0.32 µg. per litre of plasma. The mean of apparent noradrenaline levels was 2.66 µg. per litre of plasma (S.D. ± 1.12) with extremes of 0.79 and 5.49 µg. per litre.

There is suggestive evidence, to be more fully discussed later, that the fluorescence estimated as noradrenaline in plasma is produced by one or more other substances, whose condensation product shows a fluorescence ratio close to, but not identical with that shown by the condensation product derived from noradrenaline. Consequently it is suggested that values for apparent noradrenaline concentration do not represent the true value for the noradrenaline concentration in peripheral plasma.

INSULIN HYPOGLYCAEMIA

A short account of the clinical background and the pharmacological concepts relating to insulin hypoglycaemia will now be presented.

In 1922, Banting and Best successfully demonstrated for the first time, in extracts of degenerated and foetal pancreas, the presence of a substance capable of reducing the degree of hyperglycaemia and of raising the carbohydrate tolerance of diabetic (depancreatised) dogs. Also, they and their associates (Banting et al. 1922a) described characteristic effects of insulin injection in normal rabbits - hyperexcitability, hunger, thirst, and fear, progressing to convulsion and coma. Glucose injection was found to restore the animal to normal. A preliminary clinical report (Banting et al. 1922b) showing the action of insulin in lowering the blood sugar in patients with diabetes mellitus was followed by a description of the clinical symptom complex "hypoglycaemia" (Fletcher and Campbell, 1922; Banting et al. 1923).

Fletcher and Campbell described the state of hypoglycaemia in these words: "The initial symptom may be a feeling of nervousness or tremulousness, sometimes a feeling of excessive hunger, at other times a feeling of weakness or a sense of "goneness." The level at which a patient becomes aware of the fall in blood

sugar is fairly constant for that individual, although this is not always the case ..... the reaction may go no further than this of its own accord, or it may be cut short at this stage by the administration of carbohydrate. More usually it is rapidly followed by objective signs - most frequently a sweat which may be very profuse; pallor and flushing are common; sometimes a change in pulse rate. In children, this increased pulse rate is often the means of detecting hypoglycaemia; in adults the sweat is the outstanding feature. At the same time the subjective symptoms become more severe; the feeling of nervousness may become definite anxiety, excitement, or even emotional upset. Some have complained of vertigo; others of diplopia. This is the extent of most reactions: much more severe manifestations are observed with further lowering of the blood sugar. Marked excitement, emotional instability, sensory and motor aphasia, dysarthria, delirium, disorientation, and confusion have all been seen. Syncope or collapse may occur, rarely going on to a state of unconsciousness. The blood pressure has fallen in one or two cases, but more often it is well sustained. Bradycardia has occurred; convulsions have not been seen."

In this early clinical report Fletcher and Campbell suggested, as an effective means of controlling the hypoglycaemia, the injection of adrenaline 1:1000, 1 cc.

Clinical experience quickly began to suggest certain alterations in cardiovascular physiology during

insulin hypoglycaemia. Thus, in 1923, Gigon reported the death from cardiac failure of a diabetic patient who had received three doses of insulin. Reinwin (1929) observed two patients with diabetes and circulatory insufficiency, in whom insulin administration appeared to increase the degree of congestive failure. Cardiac infarction following insulin administration was reported by Joslin (1928), and Blotner (1930), while death during insulin hypoglycaemia occurred in several patients with coronary artery disease (von Noorden and Isaac, 1927).

In several instances, insulin hypoglycaemia was held to be responsible for precipitating an attack of angina pectoris (Hetényi,1926; Katz, 1929; Turner, 1930). Diminished amplitude of the electrocardiographic tracing, and inversion of the T wave during insulin hypoglycaemia were other findings (Middleton and Oatway, 1931).

Studies of cardiac output (Lauter and Baumann, 1929), employing the ethyl iodide method of Henderson and Haggard (1925), showed an increase during hypoglycaemia in one normal subject and five patients with diabetes mellitus. Ernstene and Altschule (1931), using the acetylene method of Grollman (1929), measured the cardiac output in 16 normal subjects. During hypoglycaemia there was an average increase of 29 per cent in the minute output of the heart. An increase in pulse pressure occurred, averaging 29 mm.Hg.

(73 per cent); systolic pressure increased 12 per cent, while diastolic pressure showed a 20 per cent decrease. Pulse rate increased, on average, by 15 per cent.

As stated by Ernstene and Altschule: "Clinically the changes in pulse rate, systolic and diastolic pressure and cardiac minute volume output which occur during insulin hypoglycaemia are similar to those that follow subcutaneous administration of epinephrine." (See Jensen, 1930; Euler and Liljestrand, 1927).

Boothby and Wilder (1923) found that insulin had no direct effect on the metabolic rate; they suggested that the increased metabolism recorded during insulin hypoglycaemia resulted from a spontaneous discharge of epinephrine. Similar clinical observations were no doubt largely responsible for the considerable interest shown by pharmacologists in the physiological changes accompanying insulin hypoglycaemia.

Olmstead and Logan (1923) observed signs of sympathetic stimulation in cats during insulin hypoglycaemia; Burn (1923) presented suggestive evidence that ergotamine could enhance the hypoglycaemic action of insulin in the rabbit, while Burn and Marks (1925) reported that section of both splanchnic nerves in the cat increased the hypoglycaemic action of insulin. Houssay et al. (1924), by cross-circulation experiments, demonstrated the liberation of an adrenal hormone during insulin hypoglycaemia. In 1924, Cannon, McIver and Bliss demonstrated in cats that the rate of the denervated heart was increased when the blood glucose level fell. This effect was not seen if the adrenal glands were first

denervated or removed, although other signs of sympathetic stimulation persisted, e.g., hair erection and restlessness. This work was later supported by Ernstene et al. (1935), who reported their measurements of heart rate and cardiac output during hypoglycaemia in normal cats after cardiac denervation, and following cardiac denervation plus adrenal inactivation (by removal of the right adrenal gland and denervation of the left). In the animals with denervated hearts and intact adrenal glands, insulin hypoglycaemia was accompanied by an average increase of 23 per cent in cardiac output (measured by the Fick principle with direct arterial and mixed venous sampling by cardiac puncture (28), and an increase in the heart rate of 26 per cent. In the animals with denervated heart and inactivated adrenal glands there was an average decrease of 10 per cent in cardiac output, with an average increase in heart rate of only 1 per cent during insulin-induced hypoglycaemia.

By histological examination of the adrenal medulla, a decrease in the chromaffin material was demonstrated in rats given large doses of insulin (Poll,1925). Similar findings have been reported by other workers (Gohar,1934; Ricci and Milani,1925; Hofmann,1926; Kahn,1926; Vogt,1947). The assay of adrenaline extracts also indicated adrenaline depletion during insulin hypoglycaemia (Saito, 1929), and the most recent quantitative estimations by biological assay have confirmed a fall in the adrenaline content (Burn et al. 1950; Hokfelt,1951; West,1951; Outschoorn,1952).

Udenfriend et al. (1953) have confirmed this by chemical assay. An increased adrenaline concentration in adrenal venous blood was reported by LaBarre and Houssa (1932), and Yen et al. (1933). More recently, a fall in the adrenaline concentration in adrenal venous blood has been demonstrated (Dunér, 1953) during glucose infusion.

Brandt and Katz (1933) found that human blood possesses adrenaline-like activities during hypoglycaemia, the blood of control subjects being inactive: they used as test objects the perfused rabbit ear, the isolated rabbit ileum, and the enucleated frog eye. Increased vasoconstriction in the perfused rabbit ear, produced by human blood withdrawn following insulin injection, was also reported by Meythaler and Wossidlo (1935). This was later confirmed (Meythaler et al. 1951), with the reservation that the response varied according to the constitutional type of the subject.

A pertinent observation was made by Freeman et al. (1934) who described the constriction of denervated blood vessels during hypoglycaemia. Contraction of the denervated nictitating membrane (Partington, 1936) and dilatation of the pupil (Abe, 1924; Bender and Siegal, 1939) were also noted. Also, sympathectomised animals were shown to be hypersensitive to the action of insulin (Schlossberg et al., 1933).

A rise in the urinary excretion of adrenaline, after insulin injection, was observed in human subjects by Euler and Luft (1952). Kalaja and Savolainen (1941),

using their fluorimetric technique, described an increase in estimated blood adrenaline after insulin administration.

Other workers, in many cases employing less specific techniques, have described controversial findings in regard to an anticipated increase in blood adrenaline content during hypoglycaemia. Stewart and Rogoff (1923) stated that the action of insulin did not differ in normal and adrenalectomised rabbits. Heilbrunn and Liebert (1939), using the frog perfusion technique, found a variable effect on testing blood obtained from patients undergoing insulin shock therapy. Shaw's method (1938) was employed by Tietz et al. (1940) and by Tietz and Birnbaum (1942), who observed little change in the estimated adrenaline level during the first two hours after insulin administration. Raab (1943), using the method of Shaw (1938), found an increased blood content, during hypoglycaemia, of the chromogenic material estimated as adrenaline by this method. Richter (quoted by Jones, 1939) using the same method, concluded that adrenaline was not present in significantly increased concentration in arterial blood at the period when hypoglycaemia was clinically apparent.

The most recently reported attempt to utilise a chemical method for the estimation of plasma adrenaline during insulin hypoglycaemia was by Weil-Malherbe and Bone (1952b) and by Weil-Malherbe (1953). In the first paper, the authors

employed their method as originally described (1952a), estimating adrenaline and noradrenaline together as "blood adrenaline." The 28 human subjects used for the study were undergoing insulin shock therapy; the insulin was injected intramuscularly and blood samples were withdrawn before and at intervals after injection. In contrast to the well-established pharmacological and clinical observations during insulin hypoglycaemia, Weil-Malherbe and Bone described a marked fall in "blood adrenaline" concentration in peripheral venous plasma, usually occurring within 30 minutes of intramuscular administration of insulin. The "adrenaline" level remained below pre-insulin levels (i.e. below estimated levels of 3 to 4 ug. per litre) during the period of hypoglycaemia. During hypoglycaemic coma, l-glutamate, l-arginine, glycine and succinate appeared to raise the blood adrenaline concentration, as did electrical stimulation of the brain. Although no explanation could be given for the lowered adrenaline level, Weil-Malherbe and Bone suggested that an increased rate of utilisation of adrenergic amines by the tissues, during insulin hypoglycaemia, might be responsible. Subsequent to the development of the method for differential estimation of adrenaline and noradrenaline, Weil-Malherbe (1953) confirmed these earlier observations (p.109) during insulin hypoglycaemia, and stated: "It must be assumed, therefore, that under the influence of insulin

the fixation of adrenaline in the tissues is increased, and this has been clearly demonstrated by Hokfelt (1951)."

Hokfelt, employing a combination of chemical and biological methods of assay, had shown that while the adrenaline content of the adrenal gland was reduced after insulin injection, this was accompanied by a marked increase in the adrenaline content of heart and liver.

In 1954, Holzbauer and Vogt reported their results obtained by the direct estimation of adrenaline in peripheral venous plasma during insulin hypoglycaemia. They employed a highly specific technique involving acid-ethanol extraction (Vogt, 1952), separation of adrenaline and noradrenaline from extracted plasma by paper chromatography (Crawford and Outschoorn, 1951), followed by biological assay of adrenaline (on the rat uterus) and noradrenaline (using the rat blood pressure), (29). Holzbauer and Vogt were able to demonstrate maximum values of 1 to 6.4 µg. adrenaline per litre after insulin injection in two dogs (0.2 to 2.1 units per kg.). In one human subject studied, the adrenaline concentration 45 minutes after insulin (0.24 units per kg.) was 1.8 µg. per litre. Although concentrations of noradrenaline at or above 1 µg. per litre could be recovered by their method, no noradrenaline was detected in peripheral venous plasma before, during or after hypoglycaemia.

It is apparent, therefore, that attemptsto use

chemical methods for the estimation of quantitative changes in adrenaline concentration during insulin hypoglycaemia have failed to agree with the results obtained by indirect pharmacological evidence based on signs of sympathetic stimulation, and on signs of stimulation of organs previously denervated, and with good evidence, adequately confirmed, of adrenal medullary depletion; chemical methods have also failed to confirm the convincing evidence of adrenaline release obtained by direct pharmacological assay of peripheral venous blood.

An attempt was therefore made by the author to use the ethylene diamine condensation method, modified as described from the method of Weil-Malherbe and Bone (1952a, 1953), for the quantitative assay of adrenaline in peripheral venous plasma during the hypoglycaemia induced by insulin.

METHOD

Dogs

Six studies were carried out on 4 dogs which, although not specially trained, had been previously exercised and fed by the author. On each occasion they had been fed approximately 18 hours prior to the study.

Blood for sampling (32 ml.) was withdrawn from any convenient leg vein after superficial infiltration with xylocaine. Usually two veins were used during the study, and occasionally the limbs of the dog were placed in warm water to increase blood-flow. After withdrawal of a control sample of blood, soluble insulin was injected intravenously in a dose of 1.7 to 2.4 units per kg. Three further samples of blood were drawn, and at a variable time, usually about ninety minutes after the start of the study, 5 to 6 gm. 25 per cent glucose were injected intravenously. One or two further blood samples were subsequently withdrawn during the recovery period.

From previous studies in dogs it had been found, using the chemical method of estimation employed in this study, that there was no measurable rise in the estimated plasma adrenaline concentration as a result of the withdrawal of 180 ml. blood, the approximate amount required for each insulin study.

Human subjects

Five healthy male students (ages 21 to 30) were studied in the morning following an overnight fast. Each subject was allowed to rest for 10 minutes, following which, after superficial infiltration with xylocaine, a wide bore needle with stilette was inserted into a convenient forearm vein, used for insulin injection and the withdrawal of blood samples. Arterial blood pressure was recorded by the auscultatory method, a reading being taken just before or just after the withdrawal of a blood sample.

Following withdrawal of the control sample of blood, insulin was injected intravenously. Three to four further samples were withdrawn until termination of the hypoglycaemia by the intravenous infusion of 500 cc. of 5 per cent glucose in water, supplemented by 25 cc. of 50 per cent glucose.

The three studies during which ballistocardiogram tracings were taken were carried out in a similar fashion, with the subject lying on a high frequency Starr ballistocardiograph table.

The blood samples were withdrawn into heparinised 30 ml. syringes which were placed in a container and surrounded with ice. At the conclusion of a study, each sample was placed in a chilled 50 ml. centrifuge tube, and after withdrawal of 1 ml. blood for glucose determination, the sample was centrifuged for 20 minutes at approximately 300 g. The plasma from each sample

was aspirated and divided into two portions of 7 to 10 ml. (the volume being noted) which were placed in clean test tubes to each of which was added 5 ml. sodium fluoride/sodium thiosulphate solution.

The plasma samples were covered and stored overnight at 3°C. The adrenaline estimations were performed on the day following the study, employing the reagents and method already described (pages 40-52).

The blood glucose determinations, based on the colorimetric method of Nelson (1944) were performed on the day of the study.

The basis of the method of Nelson is that glucose present in a protein-free filtrate of blood reduces copper in alkaline solution when heated. The cuprous copper thus formed reacts with arsenomolybdate reagent to give a stable blue colour. The colour is compared with that given by known amounts of glucose.

Reagents

5% Zinc sulphate.

0.3N Barium hydroxide (22.5 gms./500 ml.) filtered to remove barium carbonate, and stored in an aspirator bottle containing a soda-lime tube in the stopper.

These solutions are diluted 1 to 4, although the concentrations of zinc sulphate and barium hydroxide is less important than the fact that they exactly

neutralise each other (this is carefully checked by titration, with phenolpthalein indicator).

Copper Reagent A.

25 gms. anhydrous sodium carbonate
25 gms. sodium potassium tartrate
20 gms. sodium bicarbonate
200 gms. anhydrous sodium sulphate
Water 1,000 cc.

Copper Reagent B.

15% copper sulphate solution, to 500 cc. of which is added 0.25 ml. concentrated sulphuric acid.

Prior to use, the alkaline copper reagent is constituted by diluting 1 ml. of solution B in a mixing cylinder to 25 ml. with solution A, the solution being thoroughly mixed.

Arsenomolybdate colour reagent - 50 gm. ammonium molybdate in 900 cc. distilled water; 42 ml. concentrated sulphuric acid; 6 gm. disodium orthoarsenate in 50 cc. of water. These solutions are mixed and placed in an incubator for 24 - 48 hours at 37°C., then stored in a brown glass-stoppered bottle.

Glucose standard solutions - made from a stock solution containing pure dextrose dissolved in 0.2% benzoic acid solution, 10 mgm. per ml.; 0.5 ml., 1 ml., and 2 ml. of stock solution are diluted to 200 ml. with benzoic acid solution to give 50 mgm., 100 mgm., and

200 mgm. per cent glucose standard solutions respectively.

Preparation of protein-free filtrate (Somogyi, 1945) - In a 50 ml. glass-stoppered bottle are placed 9.5 ml., 0.3N barium hydroxide, 1 ml. of blood for glucose determination and 9.5 ml. zinc sulphate solution. After vigorous shaking the solution is filtered.

Determination of blood glucose - 1 ml. alkaline copper reagent is added to 0.5 ml. of each of the three standard solutions and to 0.5 ml. of water for blank solution, in test tubes. After mixing, a marble is placed on top of each tube which is heated for 20 minutes in a vigorously boiling water bath. The tubes are then removed and placed in water at room temperature for 1 minute. To each tube is added 1 ml. arsenomolybdate reagent, followed by 7.5 ml. water. After careful mixing by inversion, the colour densities of each solution are measured in a suitable photocolorimeter, using a 540 mμ filter.

A Beckman spectrophotometer was employed by the author, using the blank solution for the zero setting. With the reading from the 100 mgm. glucose standard solution the following calculation gives the blood glucose concentration in mgms. per cent.

$$\frac{\text{Unknown reading} \times 100}{\text{Standard reading}}$$

In preliminary recovery experiments performed by the author, consistently satisfactory recoveries of 98 - 100% of glucose, added to blood samples, were obtained.

Study 1.
(Dog 1.)

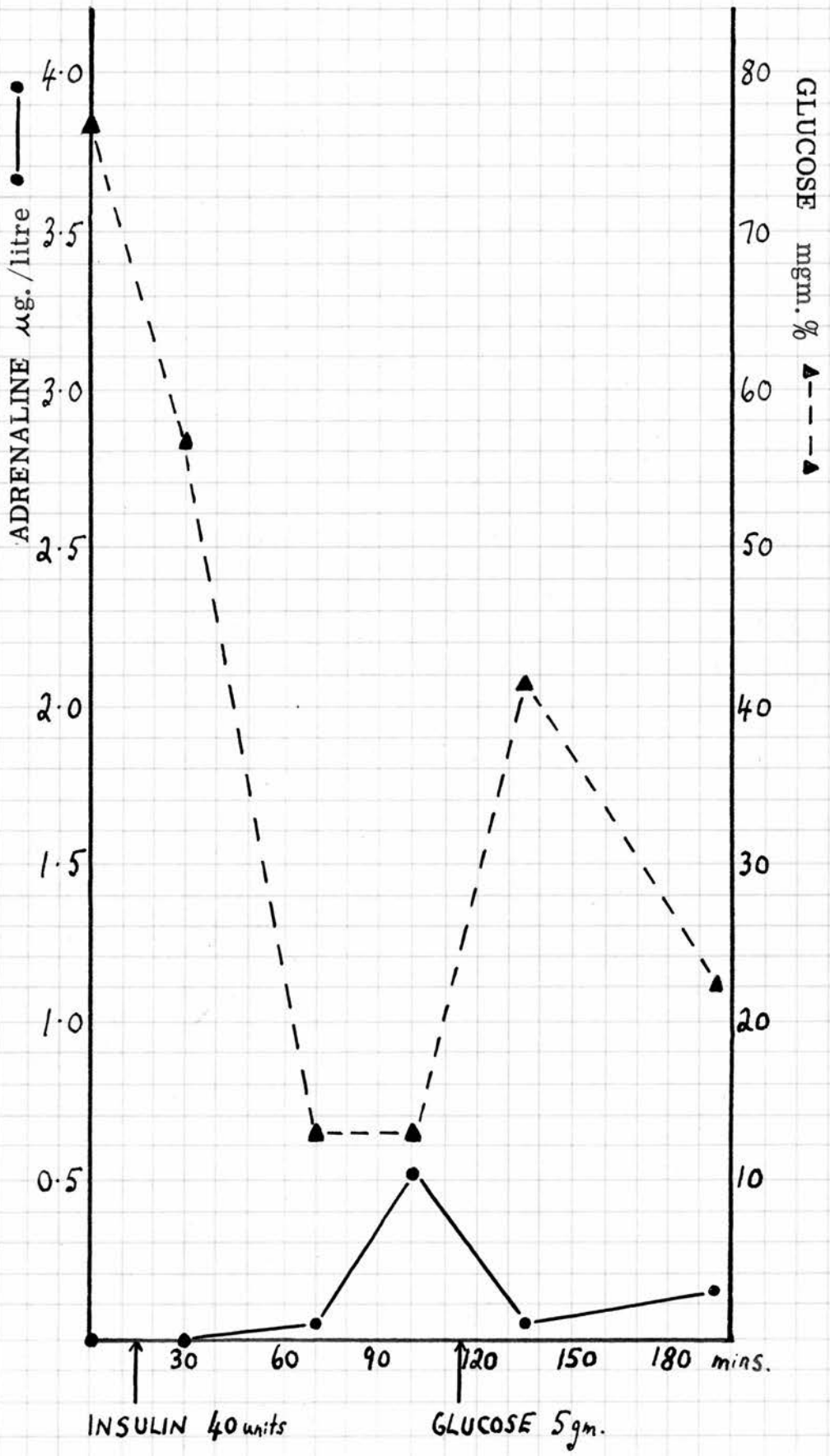


Figure 1.

Study 2.
(Dog 2.)

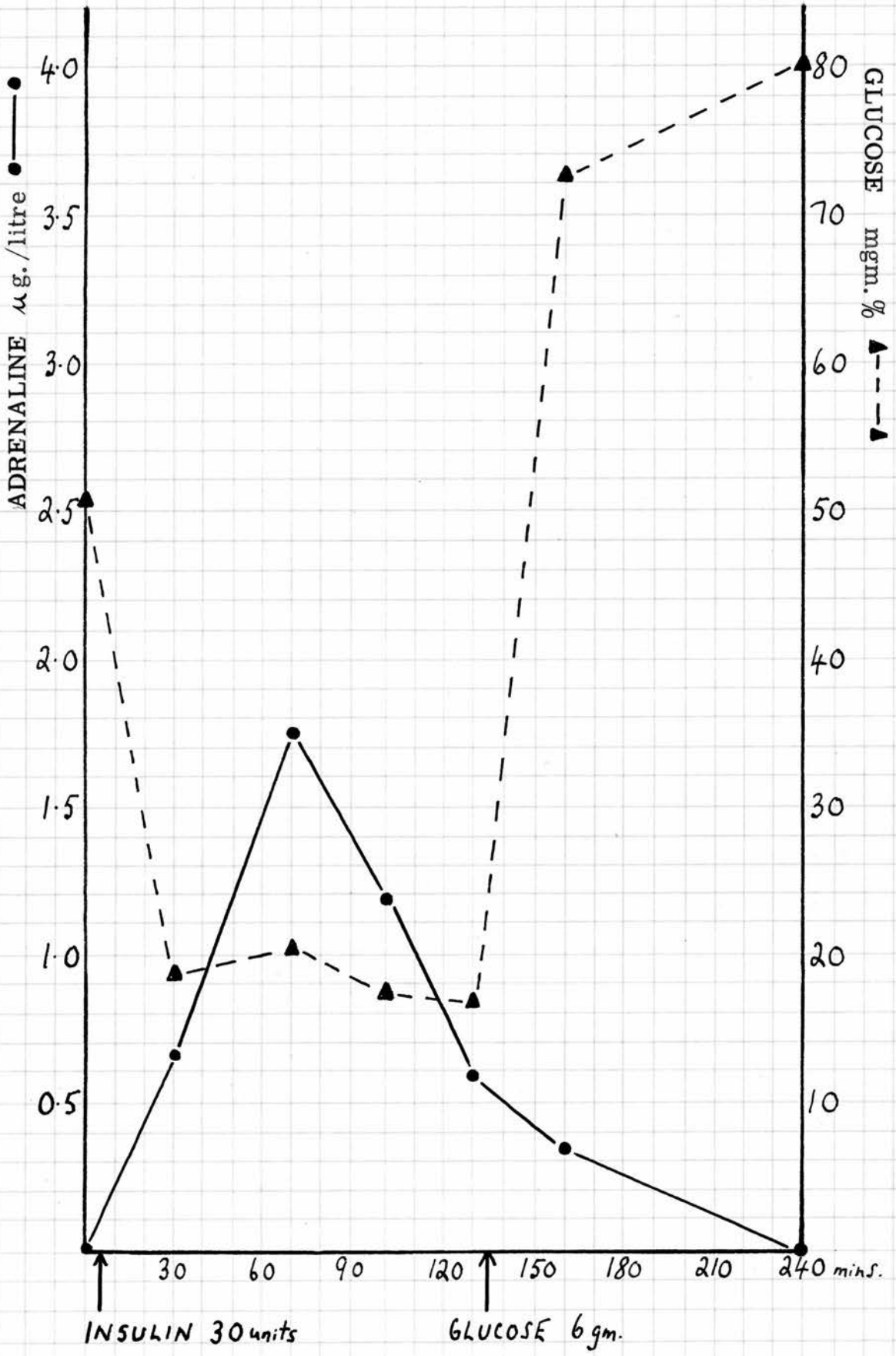


Figure 2.

Study 3.
(Dog 2.)

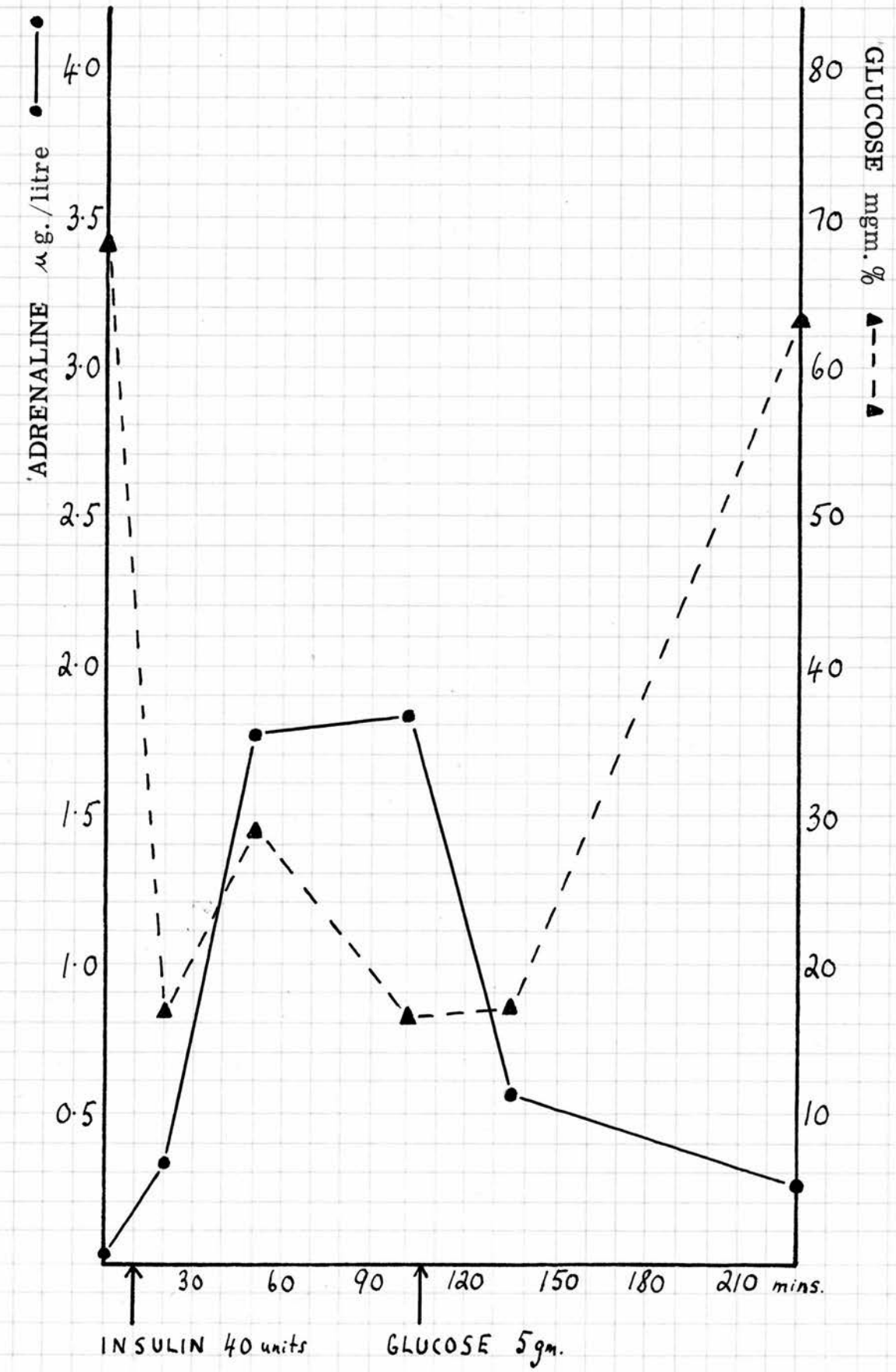


Figure 3.

Study 4.
(Dog 3.)

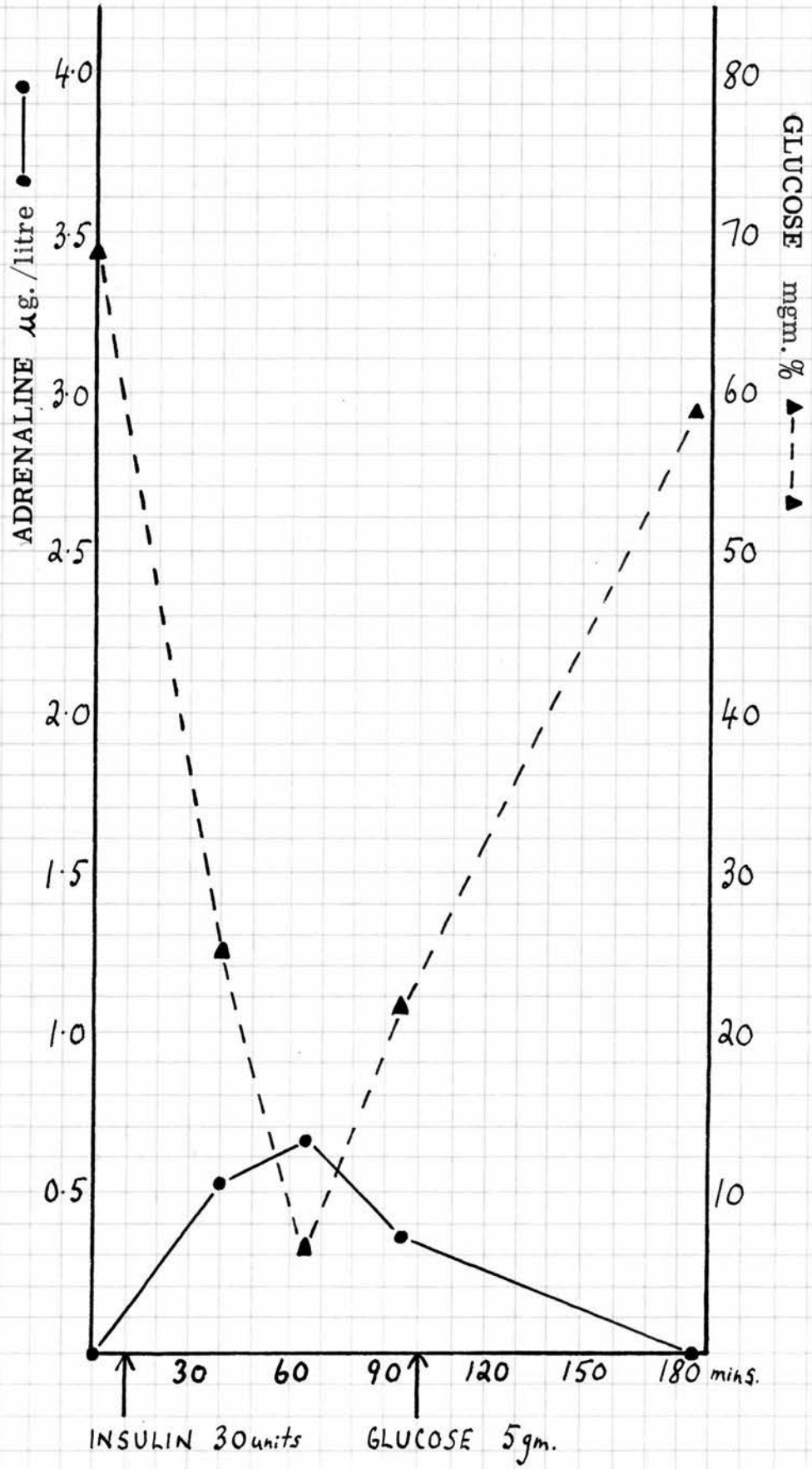


Figure 4.

Study 5.
(Dog 4.)

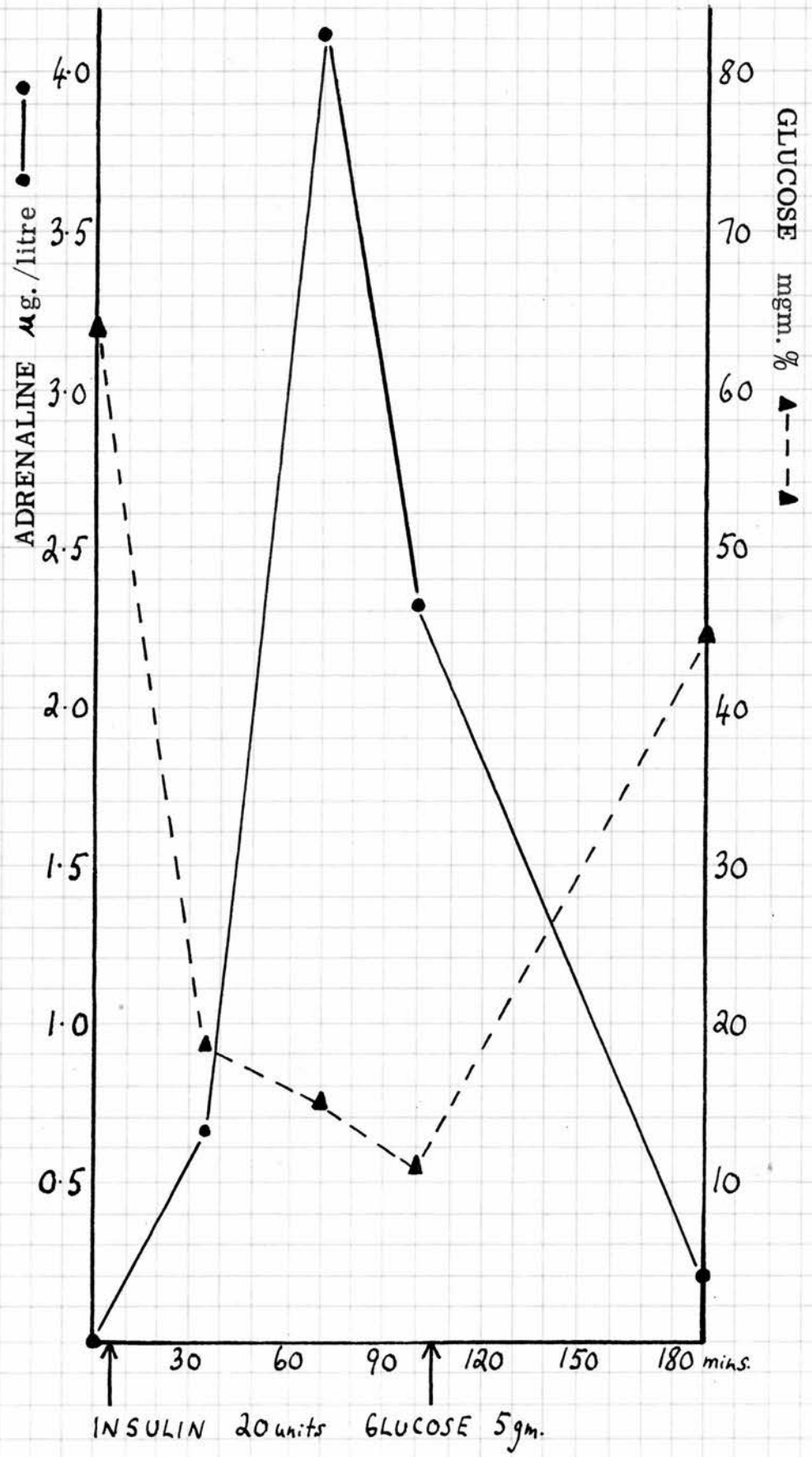
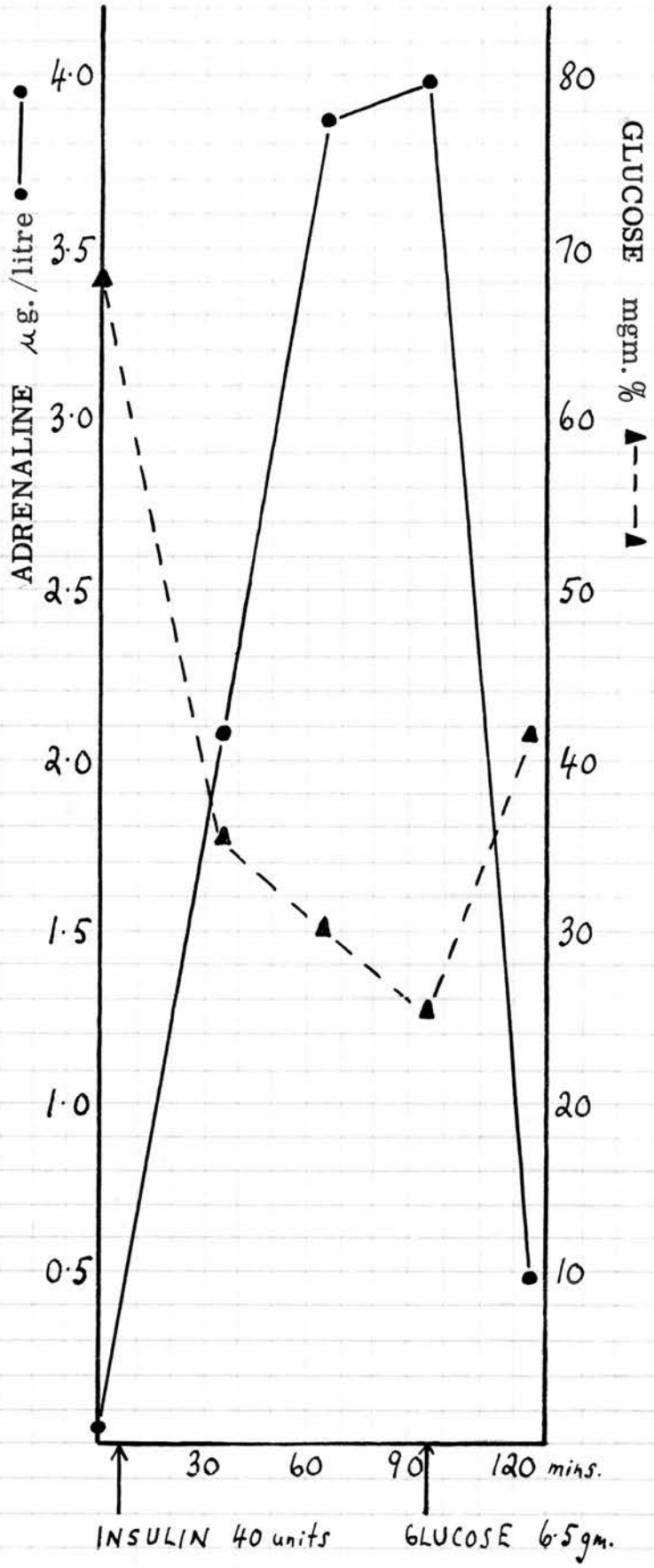
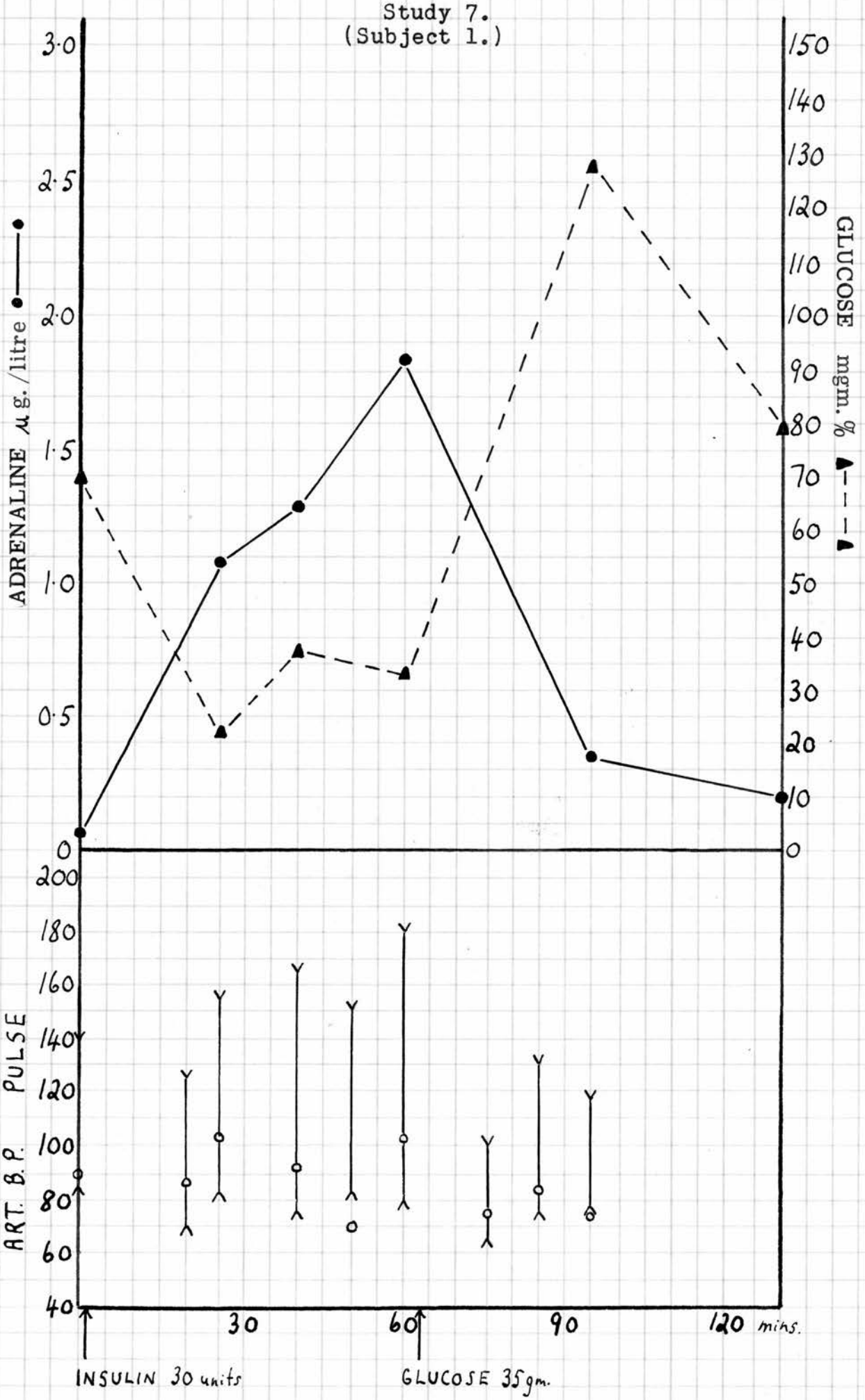


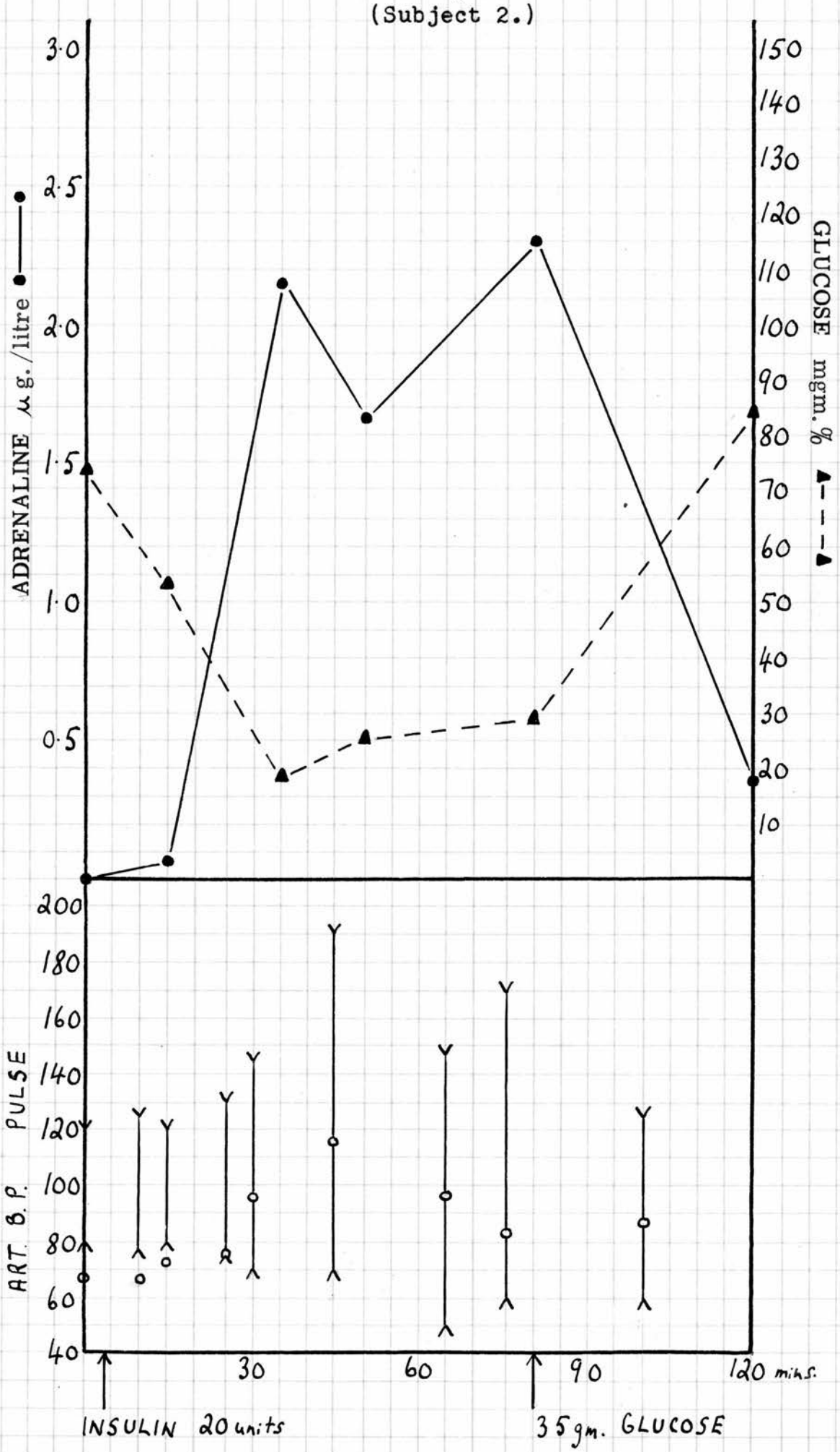
Figure 5.

Study 6.
(Dog 2.)



Fugure 6.


Study 7.
(Subject 1.)
ADRENALINE μg./litre
GLUCOSE mgm. %
3·0
2·5
2·0
1·5
1·0
0·5
0
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
200
180
160
140
120
100
80
60
40
ART. B.P.
PULSE
30
60
90
120 mins.
INSULIN 30 units
GLUCOSE 35gm.


Study 8.
(Subject 2.)
ADRENALINE µg./litre
GLUCOSE mgm. %
3·0
2·5
2·0
1·5
1·0
0·5
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ART. B.P.
PULSE
200
180
160
140
120
100
80
60
40
30
60
90
120 mins.
INSULIN 20 units
35 gm. GLUCOSE

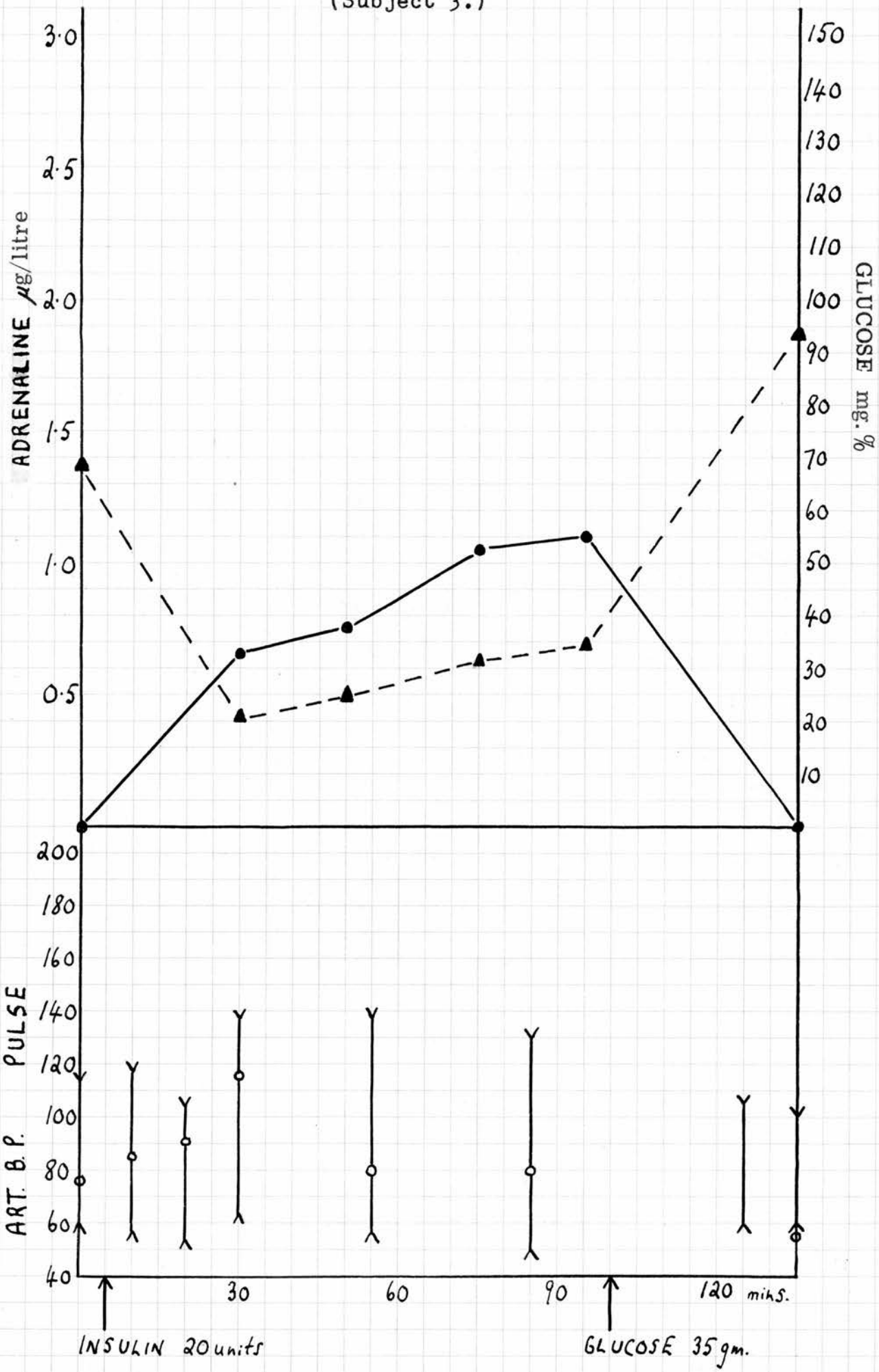

Study 9.
(Subject 3.)
ADRENALINE µg/litre
3·0
2·5
2·0
1·5
1·0
0·5
GLUCOSE mg. %
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ART. B.P.
PULSE
200
180
160
140
120
100
80
60
40
30
60
90
120 mins.
INSULIN 20 units
GLUCOSE 35 gm.

Figure 9.

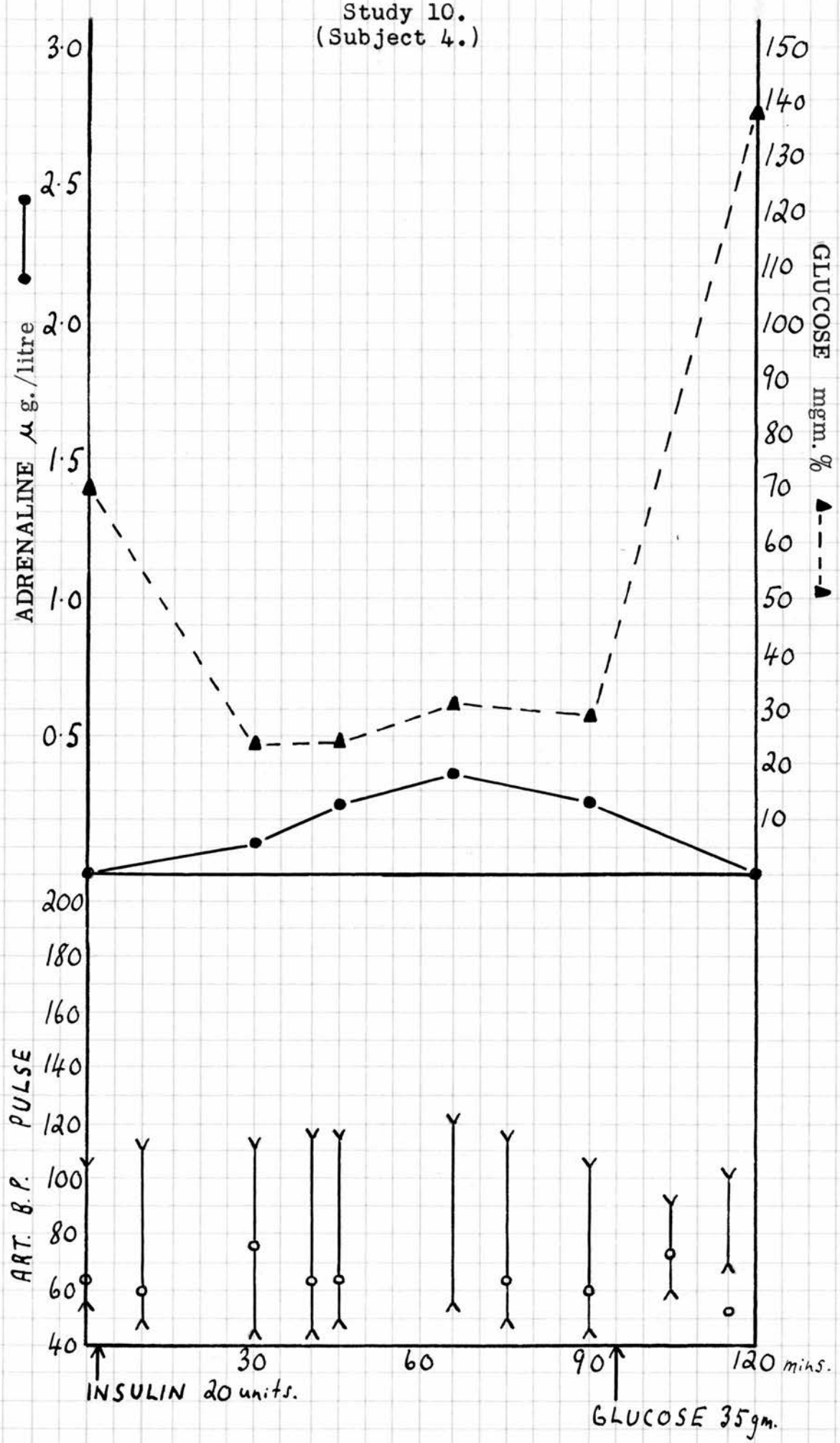
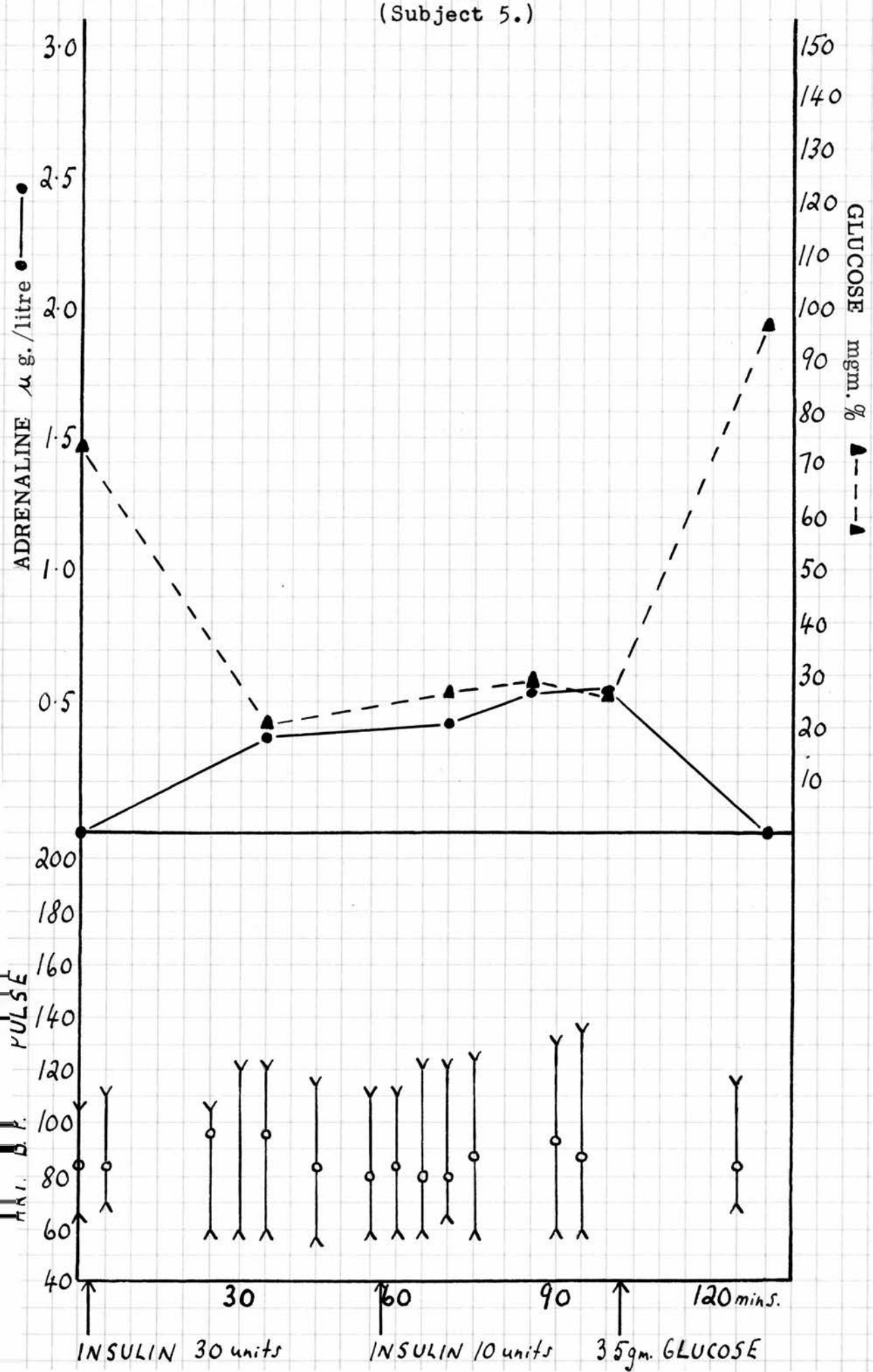

Study 10.
(Subject 4.)
ADRENALINE μg./litre
3·0
2·5
2·0
1·5
1·0
0·5
GLUCOSE mgm. %
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ART. B.P.
PULSE
200
180
160
140
120
100
80
60
40
30
60
90
120 mins.
INSULIN 20 units.
GLUCOSE 35gm.

Figure 10.

Study 11.
(Subject 5.)



RESULTS

The estimated adrenaline and blood glucose levels for each study are illustrated in Figures 1 - 11. The data from which the adrenaline concentrations were calculated is tabulated in full on pages 110 - 131.

From previous pilot studies it had been established, using the chemical method of estimation employed in this investigation, that there was no measurable rise in the estimated plasma adrenaline concentration as a result of the withdrawal of 180 ml. of blood, the approximate amount required for each insulin study. In six studies carried out on 4 dogs, there was a rise in the estimated epinephrine level in peripheral venous plasma during hypoglycaemia from virtually zero (-0.05) to a mean of 2.14 ug. per litre (range 0.51 to 4.11). Following glucose administration, the estimated adrenaline level fell to a mean value of 0.13 ug. per litre. In the 5 human subjects, the mean response was from zero (-0.14) to a maximum value of 1.22 ug.per litre epinephrine (range 0.37 to 2.30 ug. per litre), falling to 0.06 ug. per litre after glucose infusion.

Analysis of variance of adrenaline values for each study is shown in Table III (p. 132). In all studies, variance between the means of duplicate samples was significantly

greater than variance within duplicate samples (studies 2 - 11 inclusive, $p. < 0.01$; study 1, $p. < 0.02$). Within each study the difference between two adrenaline values required to achieve significance at the 5 per cent level (M.S.D.) is shown in Table IV (page 133). Combining the variance within duplicate samples gives an M.S.D. value for the entire study of 0.32 ug. per litre.

The apparent noradrenaline values for each study are shown in Table V (page 134), the numbering of each sample corresponds to that for the adrenaline values illustrated in Figures 1 - 11. A consideration of these values does not reveal any trend toward a definite or consistent increase or decrease in estimated noradrenaline following insulin administration, although in 3 studies (Nos. 1, 4, and 7) "inconsistent" variation between timed samples was significantly different from variation within duplicate samples. The mean estimated noradrenaline value for all studies was 2.32 ± 1.34 ug. apparent noradrenaline per litre.

During hypoglycaemia in the human subjects there was an increase in systolic blood pressure with a lowered or unchanged diastolic pressure. An initial increase in heart rate was usually not maintained. The typical symptoms and signs of hypoglycaemia occurred to a variable degree in every study. In the dogs, severe muscular weakness and hunger were obvious features. Generalized perspiration, palpitation, and vigorous pulsation of the

cardiac apex and carotid vessels were prominent in the human subjects. Facial pallor was not constant. Weakness, lassitude, and some degree of mental confusion were noted 10 to 15 minutes after insulin injection, lasting throughout the period of hypoglycaemia. The clinical signs of sympathetic stimulation were much less evident in subjects 4 and 5 than in the other 3 human subjects, and it is of interest that clinical observation agrees with the estimation of lower adrenaline concentrations in these 2 subjects. Subject 5 appeared to be only slightly affected by insulin 30 units, and a second intravenous injection of 10 units did not markedly increase the severity of symptoms and signs. One individual, suffering from mild coryza with nasal congestion, stated that nasal respiration became much easier during the period of hypoglycaemia.

The administration of 6 per cent glucose (30 to 40 gm.) by intravenous infusion was followed by a rapid regression of symptoms and signs accompanying hypoglycaemia. Within 30 minutes the human subjects were able to continue with their normal routine.

As has been stated, ballistocardiographic tracings were obtained in 3 human subjects during hypoglycaemia. However, in view of the indeterminate position held by ballistocardiography in the minds and opinions of both clinicians and research workers, a short account of this subject will now be presented.

BALLISTOCARDIOGRAPHY

In 1786 Parry noted in one of his patients that the body moved in time with the heart beats; he reported this in 1825. A Scotsman, Gordon (1877), attempted to record this movement, using a bed suspended from the ceiling, levers, and a smoked drum. Gordon's work was noted by Trotter (1877) and by Landois (1879), who made the first clinical observation by describing the typical record of abnormal amplitude produced by a patient with aortic regurgitation.

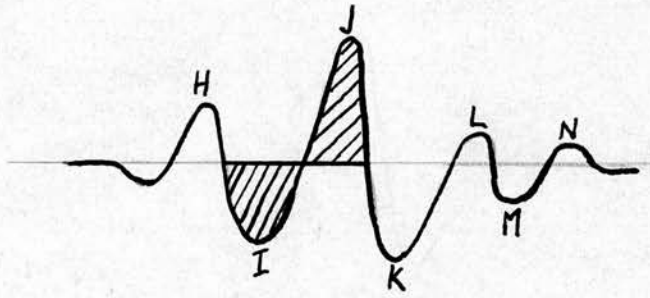
In 1905, Yandell Henderson, apparently unaware of previous work, reopened the subject, and on the famous expedition to Pikes Peak with Haldane and Douglas (to study the effects of altitude) recorded the movement imparted by a subject lying on a plank supported on corks. In 1913 Satterthwaite, a New York physician, also in ignorance of previous work, secured some records on a smoked drum. In 1922 the first attempt at electrical recording was made, but the results were unsatisfactory (Heald and Tucker, 1922).

The German geophysicist Angenheister (1928) published records obtained by placing a seismograph beside a subject lying on a rigid table. A special chair was constructed by Abramson in 1933, but difficulties were increased by having the subject in the vertical position.

Despite these early efforts, no real advances in technique or clinical application occurred until Starr

and his associates (1939) reported their original investigations. Much of the current knowledge regarding the physical and physiological aspects of ballistocardiography results from studies subsequently carried out by Starr in Philadelphia. He and his associates constructed a "high frequency" table; the records taken from subjects were called "ballistocardiograms." A subject lying on the table was free to move only in his longitudinal dimension, the movement in this direction being opposed by a strong spring. The movement was magnified by a light beam system and photographed. The modern resisted table employs an electric pick-up and amplification system, recording by a direct writer; for the studies on insulin hypoglycaemia and for the infusion experiments, the Starr table was utilised.

Newton's third law of motion states: "for every action there is an equal and opposite reaction."



Wave H is due to a small headward movement of the body caused by the movement backward of the heart and blood within it in the isometric period of systole.

Wave I is due to a sharp recoil of the body feetward as the blood is ejected into the aorta.

The J wave indicates the headward movement caused by the recoil of the aorta as the blood flows down the descending aorta.

The feetward wave K is produced by the deceleration of the column of blood in the thoracic and abdominal aorta.

The other waves, L, M, and N are after-vibrations occurring during systole.

The basic equation from which the analysis of the ballistocardiogram was initially derived was the Newtonian equation for force.

By a combination of theoretical reasoning and empiricism, a series of formulae were devised. In 1942, Cournand et al., selecting one of these, found that estimation of cardiac output gave results 18.5 per cent lower than comparable estimates based on the Fick method, employing cardiac catheterisation. Nickerson et al. (1947), who used a differently constructed "low-frequency" table allowing free movement of the subject in the longitudinal dimension, with a different formula for calculation of cardiac output, obtained fair agreement between such estimates and those using

the Fick method.

However, although many formulae were subsequently suggested and employed, doubts were entertained with regard to the accuracy of estimation of cardiac output in patients with abnormal ballistocardiograms.

As suggested personally by Starr the following formula, No. 12 (Starr, 1955) modified from Tanner (1949), was used by the author in the studies described for the estimation of cardiac stroke volume:

$$\text{Stroke volume} = 27 \times \text{S.A.} \sqrt{(21 + J)\sqrt{C} + 10}$$

where S.A. = surface area in square metres.

I and J = area of ballistocardiographic waves in millimetre seconds when I mm. deflection equals 28 gm. force. Each wave is considered as a triangle, the area therefore being 1/2 (base) X height.

C = duration of the cardiac cycle in seconds.

Starr (1955) has stated: "when body surface area or age is introduced into a regression equation including Tanner's formula the accuracy of the estimate is improved. The error is less than 10 cc. in two-thirds of the estimates; if the data of the most arteriosclerotic case are omitted this error falls to 8 cc. The error in the detection of changes of stroke volume in single persons is considerably less than this, and the method seems ideal for estimation of changes of

cardiac output due to drugs and other physiological agents. When the ballistocardiogram is abnormal in form the estimate by these formulae may give a correct result, but sometimes the result is low. On the basis of present knowledge, clear directions cannot be given which will separate the abnormal records which can properly be made the basis of an estimate of stroke volume, from those which should not be used for this purpose. This is a serious drawback to the routine use of the ballistic stroke volume method in the clinic, where so many of the older patients have ballistocardiograms to some degree abnormal in form."

Although the clinical application of information derived from ballistocardiographic records is a controversial subject, it is generally agreed that the ballistocardiograph measures cardiac force. For the evaluation of pressor drugs and other agents which affect the force of cardiac contraction, ballistocardiograph records should hold an important place.

The circulatory data obtained from subjects 3, 4, and 5 is summarised in the Tables on page 137 and was reviewed by Dr. Isaac Starr, whose cooperation and advice the author wishes to acknowledge with gratitude. The view expressed by Dr. Starr (1956) that the records closely resembled those obtained during infusion of l-adrenaline, is based on the increase in cardiac force clearly evident in the records (pages 165), and on the

estimates of cardiac stroke volume obtained by calculation (page 137). During insulin hypoglycaemia, therefore, when the adrenaline concentration in peripheral venous plasma is increased, as measured by the ethylene diamine condensation method, there is at the same time an increase in cardiac force and systolic blood pressure and a decrease in diastolic pressure, physiological changes which closely resemble those attributed to adrenaline by other workers (Goldenberg at al. 1948; Barcroft and Starr, 1951).

At the conclusion of these studies on insulin hypoglycaemia in human subjects, it also seemed of interest to attempt a "physiological calibration," i.e. the correlation of known amounts of l-adrenaline, infused intravenously, with plasma levels of adrenaline as estimated by the ethylene diamine condensation method. Two studies were carried out by the author, using subjects 3 and 4 of the insulin study (pages 138 - 143).

"Suprarenin" brand of l-epinephrine tartrate containing 1 mg. adrenaline base per ml. was employed. An electrically driven shaft, coupled to a 50 ml. syringe and accurately calibrated to deliver infusion rates of 1 to 4.5 ml. per minute was used; l-adrenaline was added to normal saline solution to give concentrations of 1 or 2 ug. per ml. A forearm vein was used for infusion, blood samples being withdrawn from the opposite arm. Arterial blood pressure, estimated by

auscultation, and ballistocardiographic tracings, were recorded at the time of withdrawal of samples, with the infusion still running. Plasma samples were not withdrawn until equilibrium had apparently been reached between infusion, tissue uptake, and diffusion or destruction of adrenaline, as judged by the maintenance of steady blood pressure readings.

The results of the two studies are shown on pages 142-143. The findings are of considerable interest in that they give some indication of the adrenaline concentration in peripheral venous plasma at which physiological effects are present. The infusion of 1 µg. per minute, 0.01 µg. per kg. per minute, produced no change in systolic blood pressure, although the diastolic pressure fell and there was an increase in pulse rate; the estimated adrenaline level in peripheral venous plasma was 0.04 µg. per litre. 2 µg. per minute, 0.04 µg. per kg. per minute, administered to the other subject had little apparent effect on the circulation, although the estimated adrenaline concentration, 0.42 µg. per litre, was higher than that produced by infusion of 3 µg. per minute. This is difficult to explain, but may well be attributable to variations in recovery of adrenaline by the method of estimation.

When an infusion of 3 µg. per minute was employed (0.06 and 0.04 µg. per kg. per minute), both subjects showed well marked increases in systolic pressure and

pulse rate, with a fall in diastolic pressure. These features, together with the ballistocardiographic records, which indicated an increase in cardiac force, as previously described by Barcroft and Starr (1951), were closely similar to those observed during the studies on insulin hypoglycaemia in the same subjects. The adrenaline concentrations in peripheral plasma in the two subjects infused with 3 µg. l-adrenaline per minute (0.25 and 0.34 µg. per litre) were in fair agreement.

It may be hazardous to draw conclusions from these restricted data. However, the suggestion could be made that a level of 0.3 to 0.5 µg. adrenaline per litre of peripheral venous plasma may be required before definite physiological effects are apparent. It is of interest that the infusion of 0.04 and 0.06 µg. per kg. per minute (corresponding to 3 µg. per minute in the two subjects studied), is in the same range as that reported by several workers for the normal "resting" output of adrenaline from both adrenal glands (Wada and Kanowoka, 1935; Kaindl and Euler, 1951; Dunér, 1953; Houssay and Rapela, 1953).

It was recently reported by Ludemann (1955) that following the rapid intravenous injection of 450 µg. adrenaline, the concentration in peripheral arterial blood fell rapidly to control levels within 2 minutes 45 seconds. Although it is currently believed that there is a "resting" secretion of adrenaline from the adrenal medulla, the liberated adrenaline is probably rapidly

destroyed by amine oxidase, catechol oxidase, or other enzymes, or inactivated by tissue diffusion. To produce and maintain effects on cardiac output and blood pressure may require a rate of secretion of adrenaline into the adrenal veins other than the 0.04 to 0.06 µg. per kg. per minute required to produce the same effects when infused into the antecubital vein.

An infusion of 6 µg. per minute (0.11 µg. per kg. per minute) produced the typical gross circulatory effects of adrenaline, with an increase in arterial blood pressure to 136/62 and an estimated adrenaline level of 1.40 µg. per litre. Although many factors may be concerned in the regulation of blood pressure during insulin hypoglycaemia, it is of interest that in the same subject a comparable blood pressure, 130/50, was accompanied by an estimated adrenaline level of 1.10 µg. per litre.

In summary, it can be stated that the blood pressure changes and the ballistocardiographic records obtained both during hypoglycaemia and during infusion of l-adrenaline provide substantial support for the presence of an increased level of adrenaline in peripheral venous plasma in the range of concentrations as estimated by the ethylene diamine condensation method, during the hypoglycaemia induced by insulin.

DISCUSSION
and an appraisal of the
ethylene diamine condensation method

The need for an accurate and specific method for the chemical estimation of adrenaline in blood has been appreciated for 50 years, although attempts to use chemical techniques for blood analysis have failed to agree with the results obtained by more specific methods of biological assay. The two distinct problems involved - the preliminary separation of adrenaline from interfering substances in plasma, and the necessity for an adequately sensitive and specific method of colorimetric or fluorimetric estimation - have appeared insurmountable. It is clear that before chemical methods can be accepted and employed in the pharmacological and clinical laboratory, they must either agree with established physiological concepts, or prove convincingly that they are in error.

Although a substantial body of pharmacological evidence supports the occurrence of adrenaline secretion during hypoglycaemia, this has been challenged by results obtained with the ethylene diamine condensation method (Weil-Malherbe and Bone, 1952b; Weil-Malherbe, 1953). In the course of a general evaluation of the method of Weil-Malherbe and Bone (1952a, 1953) the author embarked

on an attempt to estimate adrenaline concentrations in peripheral venous plasma following the administration of insulin; several possible results might thereby accrue. If successful, the study would confirm, for the first time by a chemical method of blood analysis, the established concept of an increased adrenaline blood content during hypoglycaemia; for comparison, there would be available the data obtained by the direct estimation of adrenaline in peripheral venous plasma by means of a specific technique based on biological assay (Holzbauer and Vogt, 1954). At best, a successful result would instil confidence in the reliability of the ethylamine diamine method; if unsuccessful, something would be learnt which might offer an explanation for the contradictory results achieved with the ethylene diamine method by Weil-Malherbe and Bone (1952b). At worst, the method might be finally discarded as a means of estimating adrenaline in peripheral blood.

With this in mind, the ethylene diamine condensation method as employed for the studies described in this thesis will be reconsidered.

The use of aluminium oxide or hydroxide appears to assure adequate specificity for the adsorption from plasma of dihydroxyphenyl derivatives (Shaw, 1938; Raab, 1943; Lund, 1949c; Weil-Malherbe and Bone, 1952b; Manger et al, 1954; Aronow and Howard, 1955; Ludemann et al. 1955; von Euler and Orwen, 1955).

The condensation reaction with ethylene diamine (Natelson et al. 1949) on which the method of Weil-Malherbe and Bone depends, requires the presence of the catechol nucleus. From the number of possible interfering substances listed on page 108, it is apparent that two compounds must be closely considered, viz. 3:4-dihydroxyphenylacetic acid (catechol acetic acid) and hydroxytyramine (dopamine).

Recently published work by von Euler and Floding (1955) suggests that catechol acetic acid is responsible for the greater part of the fluorescence produced by the condensation of alumina eluates of urine extracts with ethylene diamine. The biologically active catechol amines - hydroxytyramine, adrenaline and noradrenaline - were found by Euler and Floding to account for only a small fraction of the total catechol compounds present in urine.

In recovery experiments carried out in this laboratory (Valk, 1956; page 108), catechol acetic acid, when condensed with ethylene diamine, was found to have a fluorescence ratio (on the two secondary filters) which differs from that shown by noradrenaline by 10% or less. From a comparative assay of plasma samples obtained from dogs during haemorrhagic shock, by the ethylene diamine method and by the method of Lund (1950), carried out in this laboratory (Valk and Price, 1956), adrenaline values

as estimated were found to be in excellent agreement - 1.78 µg/litre by the ethylene diamine method, 1.84 µg/litre by Lund's method. Estimated noradrenaline levels by the latter technique, however, were only 30% of those attributed to noradrenaline by the ethylene diamine condensation method. The method of Lund (1949c; 1950) requires the formation of fluorescent trihydroxy-indole derivatives from adrenaline and noradrenaline (page102). Catechol acetic acid is incapable of indole ring closure, as can be ascertained from the requirements listed on page 31, and would therefore escape detection by the method of Lund.

If present in peripheral venous blood, catechol acetic acid would be responsible for part of the fluorescence attributable to noradrenaline by the method of Weil-Malherbe and Bone. Furthermore, since the method uses adrenaline and noradrenaline standard solutions for the construction of a simultaneous equation for each secondary filter used, the presence of catechol acetic acid in plasma eluates would have the effect of introducing an error, constant or variable, into the estimation of adrenaline concentration.

This suggests that methods based on condensation of plasma eluates with ethylene diamine are insufficiently specific for the estimation of noradrenaline in blood. This is confirmed by the work of Holzbauer and Vogt (1954),

who failed to detect any noradrenaline in peripheral venous blood in 2 dogs and one human subject, although the sensitivity of their specific biological method allowed the estimation of 1 µg. noradrenaline per litre. It should be emphasised that no conclusions have been drawn from the apparent noradrenaline values estimated in the course of the studies on insulin hypoglycaemia.

Hydroxytyramine has been identified in urine (Holtz et al. 1942; Holtz et al. 1947; Euler et al.1951), in adrenal extracts (Goodall, 1950a; Shepherd and West, 1953), and in mammalian heart (Goodall, 1950b). The fluorescence ratio produced by hydroxytyramine (1.13) on condensation with ethylene diamine is close to that of adrenaline (0.58) (Valk, 1956; page 108). Although the presence of hydroxytyramine in blood has not been demonstrated, and while its biological activity is weak in comparison with that shown by adrenaline, the possibility exists that it may act as an interfering substance in the estimation of low plasma levels of adrenaline by the ethylene diamine condensation method.

By the method of Lund, hydroxytyramine produces only a weak fluorescence (Euler and Floding, 1955). The formation of fluorescent trihydroxyindole compounds appears therefore to be more specific for adrenaline and noradrenaline than is the ethylene diamine condensation reaction. It should be mentioned, nevertheless, that the

method of Lund allows the estimation of isopropylnor-adrenaline, recently tentatively identified in very small amounts in adrenal extracts (Lockett, 1954). With the ethylene diamine method, isopropylnoradrenaline appears to have a closely similar fluorescence ratio (0.73) to that of adrenaline (0.58) (Valk 1956; page 108), but until its presence in tissue extracts or body fluids is confirmed, its possible role as an interfering substance can hardly be considered.

Discussion of Results

Preliminary use of the ethylene diamine condensation method (Weil-Malherbe and Bone, 1953) indicated an average adrenaline concentration in ambulant human subjects of 1.32 µg. per litre of peripheral venous plasma. Subsequently, other workers have reported normal levels of 0.14 ± 0.21 (Manger et al. 1954) and 0.4 ± 0.2 (Aronow et al, 1956) µg. per litre. In this laboratory, estimations on ambulant normal volunteers have indicated an average level of 0.097 ± 0.14 µg. adrenaline per litre.

In this study on insulin hypoglycaemia, with each human subject at complete physical rest and apparently at ease, virtually no adrenaline was detectable in peripheral venous plasma, prior to insulin injection. Holzbauer and Vogt (1954), employing a specific technique based on the biological assay of adrenaline on the rat uterus, found less than 0.06 µg. per litre in one human subject at rest; in two trained dogs, normal levels were below 0.25 µg. per litre. Following insulin administration in dogs, the maximum adrenaline concentrations (0.51 - 4.11 µg. per litre) as estimated in this study are closely comparable with the range of maximum values found by Holzbauer and Vogt (1 - 6.4 µg. per litre). Also their figure of 1.8 µg. per litre, obtained in one human subject, falls within the range of maximum adrenaline

concentrations (0.37 - 2.30 µg. per litre) estimated in the 5 human subjects used during this study.

For the quantitative estimation of adrenaline release during insulin hypoglycaemia, therefore, there appears to be good agreement between a specific method based on biological assay and the fluorimetric technique based on condensation with ethylene diamine. Further support for the presence of an increased level of adrenaline, in the range of concentrations as estimated in peripheral venous plasma during hypoglycaemia has been provided by the blood pressure changes and ballisto-cardiographic records obtained both during hypoglycaemia and by infusion of l-adrenaline.

An outstanding issue, since the first use of the ethylene diamine method (Weil-Malherbe and Bone, 1952a, 1953), has been the disparity between normal plasma values of adrenaline and noradrenaline as estimated by this chemical method and by established methods of pharmacological assay (e.g. Holzbauer and Vogt, 1954). An explanation for the high "normal" levels of adrenaline (1 - 1.5 µg. per litre) and noradrenaline (5 µg. per litre) in peripheral venous blood, reported by Weil-Malherbe and Bone (1953) has not been and is not available; these high "normal" levels are especially controversial in view of the much lower "normal" values of adrenaline determined by other workers utilising a basically similar method (Manger et al. 1954; Valk and

Millar, 1955; Aronow, 1956).

The controversial findings reported by Weil-Malherbe and Bone (1952b,1954) and Weil-Malherbe (1953, 1955b) in regard to decreased adrenaline levels during insulin hypoglycaemia have not been substantiated by the author's studies reported in this thesis. Since the apparent normal adrenaline levels estimated by Weil-Malherbe and Bone (1953) are 1 - 1.5 µg. per litre, it is possible that a true rise in adrenaline concentration after insulin injection would be concealed, which implies that the apparent adrenaline estimated by these workers is in fact another substance. Apart from the statement of his own results, the author is unable to provide an answer to the question asked by pharmacologists and expressed in a recent annotation (British Medical Journal,1956) on the nature of the substances estimated as adrenaline and noradrenaline by the ethylene diamine method employed by Weil-Malherbe and Bone.

The occurrence of negative values for adrenaline. This has been a disturbing feature, recurring on several occasions in the course of the adrenaline estimations performed by the author for the studies described in this thesis. Since the calculation of adrenaline and noradrenaline concentrations in plasma samples depends on the solution of simultaneous equations based on the relative fluorescence of ethylene diamine condensates of plasma eluates and adrenaline and noradrenaline

standard solutions, the occurrence of occasional negative adrenaline values may be regarded as intrinsic to the method of calculation. Variations in the fluorescence of reagent blanks and, as already mentioned, the probable existence in plasma eluates of a substance the fluorescence ratio of which is close to but not identical with that shown by noradrenaline, are other factors which may play a part in the estimation of negative values for adrenaline.

However, substantially negative values for adrenaline appear to be due to the introduction during the technical procedure of non-specific contaminating substances, in that their fluorescence spectra are revealed predominantly on the green 500 mμ secondary filter. In fluorescence ratio, therefore, these substances to some extent resemble noradrenaline. The presence of non-specific contamination in control samples is shown by high fluorescence readings on both secondary filters, by very high apparent noradrenaline values, and by substantially negative adrenaline values, e.g. samples 1a and 1b of study 1 (page111); sample 1b of study 8 (page125); sample 5a of infusion 2 (page141).

While it seems certain that such spurious fluorescence indicates contamination, the nature of this contamination has not been established; consequently, no values have been discarded from these studies, and the

temptation to call the negative adrenaline values "zero" has been rejected. In consequence, the unfortunate effect produced by taking the mean estimation of a contaminated and non-contaminated plasma sample has been brought to the fore,e.g. samples 1a and 1b of study 8; sample 5a and 5b of infusion 2, page 141.

It is possible that non-specific contamination may be produced by a substance released from plasma under certain conditions; such a substance, if adsorbed on aluminium oxide and carried into the plasma eluate, might be responsible for spurious fluorescence. For adsorption on alumina and condensation with ethylene diamine, the substance would require to be a dihydroxyphenyl derivative, and it could be argued that it might be a degradation product of adrenaline or noradrenaline released in plasma before adsorption, or formed during the adsorption procedure. The necessity for scrupulous attention to cleanliness of all glassware is clearly inherent in the satisfactory performance of any fluorimetric method for the estimation of minute amounts of an endogenous chemical substance. In the laboratory employed by the author, all glassware was thoroughly cleaned with 10% dichromate/sulphuric acid cleaning solution, washed with tap water several times, and finally rinsed out with distilled water, before drying in an electrical oven. These cleaning procedures, and all the technical procedures involved in blood glucose and

blood adrenaline estimations, were carried out personally by the author.

Negative adrenaline values, as estimated from the mean of contaminated and non-contaminated duplicate control samples of plasma withdrawn during the insulin studies,may suggest that the subsequent measurement of zero levels of adrenaline indicates a quantitative increase in adrenaline concentration. This is very questionable unless both samples show negativity, e.g. samples 1a and 1b of study 4 (page 117), in which substantially negative values for adrenaline also occur in the final samples 5a and 5b. In this case it appears likely that the estimation of a mean value of 0.52 µg adrenaline per litre in sample 2 indicates an increase of 0.78 µg adrenaline per litre.

The analysis of variance of apparent noradrenaline values (Table VI) shows that variation between timed samples (which could be accounted for by catechol acetic acid or by non-specific contamination) was not significantly different from variation within duplicate samples except in the case of 3 studies (Nos. 1, 4 and 7). In study 7 (page 134) it is very suggestive that non-specific contamination was responsible for the high apparent noradrenaline value for sample 2; estimated noradrenaline values for the other samples in this study agree quite consistently throughout the study. In study 1 (page 134)

the variation is inconsistent, while in study 4 there is a marked fall in apparent noradrenaline in the final two samples. It is clear from the analysis of variance (pages 135 - 136) that the ethylene diamine method (as employed for these studies) is quite inaccurate for the estimation of apparent noradrenaline, apart from the fact that it is almost certainly non-specific for the determination of the true noradrenaline value in plasma samples.

This discussion has also emphasized the disturbing features of negative values for adrenaline as estimated in control samples. Where samples contain considerable quantities of adrenaline, however, the presence of contamination does not necessarily affect the estimated adrenaline value. In samples 3a and 3b of study 6 , for example (page 121), the adrenaline values as estimated are in close agreement (3.80 and 3.95 ug. per litre) while the noradrenaline values differ considerably (1.33 and 4.71 ug. per litre) (page 134). Samples 3a and 3b of study 4 present similar features, noradrenaline values of 7.01 and 4.07 ug. per litre being matched by adrenaline values of 0.53 and 0.76 ug. per litre (page 117).

This suggests that when adrenaline is present in a "contaminated" plasma sample, its presence can still be revealed accurately, within the limits of each individual study.

An important factor affecting accuracy is the wide variation in recovery at low plasma levels of adrenaline (page 107). In the case of duplicate plasma samples where one sample shows a higher adrenaline value, it seems likely that the lower value results from inadequate recovery. Although the standard deviation of recovery is wide, the available literature does not record figures for recovery of adrenaline at these low levels by a chemical method. (Another factor affecting accuracy and limiting sensitivity at low levels of adrenaline is the variation in fluorescence of reagent blanks and standard solutions).

From the author's experience, the likelihood is not great that the ethylene diamine condensation method, as described for these studies, overestimates the true adrenaline level. Furthermore, the values for adrenaline estimated during the studies described have not been corrected for the 20% loss during adsorption of catechol amines on a column of alumina.

It is probable that the estimation of zero or negative adrenaline values does not preclude the existence of small amounts of adrenaline in plasma samples. This is supported by a consideration of the minimum significant changes in adrenaline level for each study, shown in Table IV, and from the figure of 0.32 µg adrenaline per litre for the entire study.

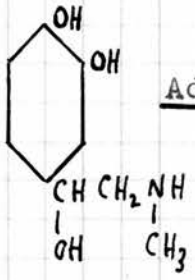
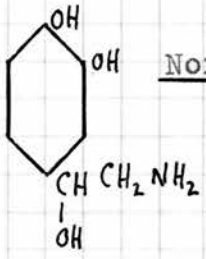
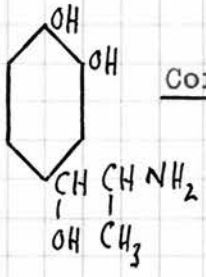
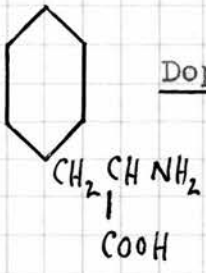
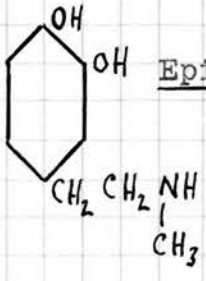
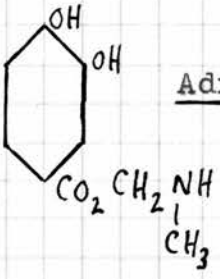
For the estimation of small changes in adrenaline concentration, for the accurate assay of adrenaline values close to zero, for the determination of absolute levels of adrenaline in peripheral venous plasma, the ethylene diamine method does not appear sufficiently accurate. However, from a consideration of the values for the minimum significant change in adrenaline concentration throughout studies 10 and 11 (0.13 and 0.07 µg per litre respectively), the possibility cannot be excluded that in favourable conditions minimal changes in adrenaline concentration could be convincingly detected by this method.

In face of the limitations of the ethylene diamine method for the estimation of plasma levels of adrenaline close to zero, and of the probable existence in peripheral venous blood of contaminating catechol degradation products such as 3:4 dihydroxyphenyl acetic acid, it is possible that the ethylene diamine reaction, because of its excellent sensitivity, may in the future find more useful application in the pharmacological laboratory for the estimation of adrenaline and noradrenaline when these substances can be separated from plasma by means of paper chromatography.

From the results obtained in the studies described in this thesis, it is concluded that although the ethylene diamine condensation method is inaccurate and

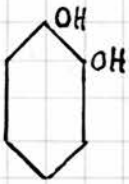
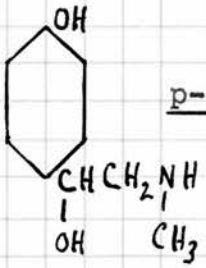
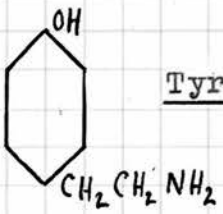
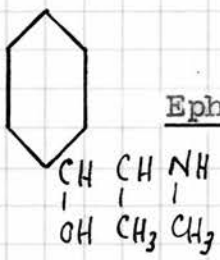
non-specific for noradrenaline, it can be suitably employed for the quantitative estimation of moderate or gross changes in the concentration of adrenaline in peripheral venous plasma.

TABLE I.

	Concentration in μg/ml giving equivalent colour after alkali treatment.	Proportional increase in colour due to alkali.
 Adrenaline	1	5
 Noradrenaline	16	No Change.
 Corbasil	12	No Change.
 Dopa	12	No Change.
 Epinine	13	No Change.
 Adrenalone	30	No Change.

Shaw (1938).

TABLE I cont'd.

	Concentration in µg/ml giving equivalent colour after alkali treatment.	Proportional increase in colour due to alkali.
 Catechol	100	Diminishes.
 p-Sympatol	2000	5
 Tyramine	No Colour.	—
 Ephedrine	No Colour	—
Glutathione	50	No Change.
Ascorbic Acid	500	Diminishes.
Glyceraldehyde	1000	Diminishes.

Shaw (1938).

Authors	Method	Sensitivity ug. %.	Peripheral Venous Blood ug. %.	
Battelli, 1902a	Ferric chloride	3000	Increased	(dog)
Wiesel & Schur, 1907	"			(man)
Stuber, Russmann & Proebsting	Iodate sulphanilic acid - mercuric chloride	2	50	(man)
Brandt & Katz, 1933	"		0	(man)
Crocetta, 1933	"		600	(man)
Dogliotti & Crocetta,1933	"		250-330	(man)
Viale, 1930,1933	"	3	200-250	(man,dog)
Viale & Crocetta, 1933	"		70-250	(dog)
Macchiarulo, 1935	"		130-200	(foetus)
Konschegg & Monauni,1936	"		50	(man)
Kobayashi, 1935	Phosphotungstic acid		7	(rabbit)
Whitehorn, 1935	Arsenomolybdic acid	2	0	(cat)
Dopy & Weisinger,1938	"	0.2	0-0.4	(guinea pig)
Sarfy, 1938,1939	"		2 (pigeon, guinea pig)	

Author	Method	Sensitivity ug. %.	Peripheral Venous Blood ug. %.	
Shaw, 1938	Arsenomolybdic acid	0.2	1.6-2 5	(man) (rabbit)
Giordano & Zeglio,1938,1939	"		20-586	(man)
Tietz,Dornheggen & Goldman, 1940	"	10	0-120	(man)
Bloor & Bullen, 1941	"	0.1	20-50	(man,dog)
Raab, 1943	"		12.3-15.6	(man)
Kobro, 1946	"	1	2.2-7.9	(man)
West, 1947	"	1	10	(rabbit)
von Euler, 1927	Methylene blue			(man)
von Euler, 1933	"	0.0000001	0.0001	(man,rabbit)
von Euler & Holmquist,1934	"		increased	(rabbit, hedgehog)
Bross & Kubikowski,1935	"		0.003	(man)
Konschegg & Monauni,1938	"			(man)
Koreff & Bender, 1940	"		0.02-0.4	(man)
von Hueber, 1940	Fluorescence	25	increased	(man)
Kalaja & Savolainen,1941	"	1	6-12	(man)
Lehmann & Michaelis,1942a,b,	"	4	71-468	(man,cat,dog)

Author	Method	Sensitivity ug. %.	Peripheral Venous Blood ug. %.	
Jorgensen, 1945	Fluorescence	1	4-10	(man)
Jorgensen, 1948	"	1	7.4	(rabbit)
Staub & Klingler, 1945	"		0	(man,rabbit)
von Porat, 1946	"		0	(man)
von Euler & Schmiterlöw, 1947	"		0	(man,cow)
West, 1947	"	1	10	(rabbit)
Bloch, 1948	"		0	(man)
Pekkarinen, 1948	"	0.5-1	0	(man, rabbit, cows, calves, dogs)

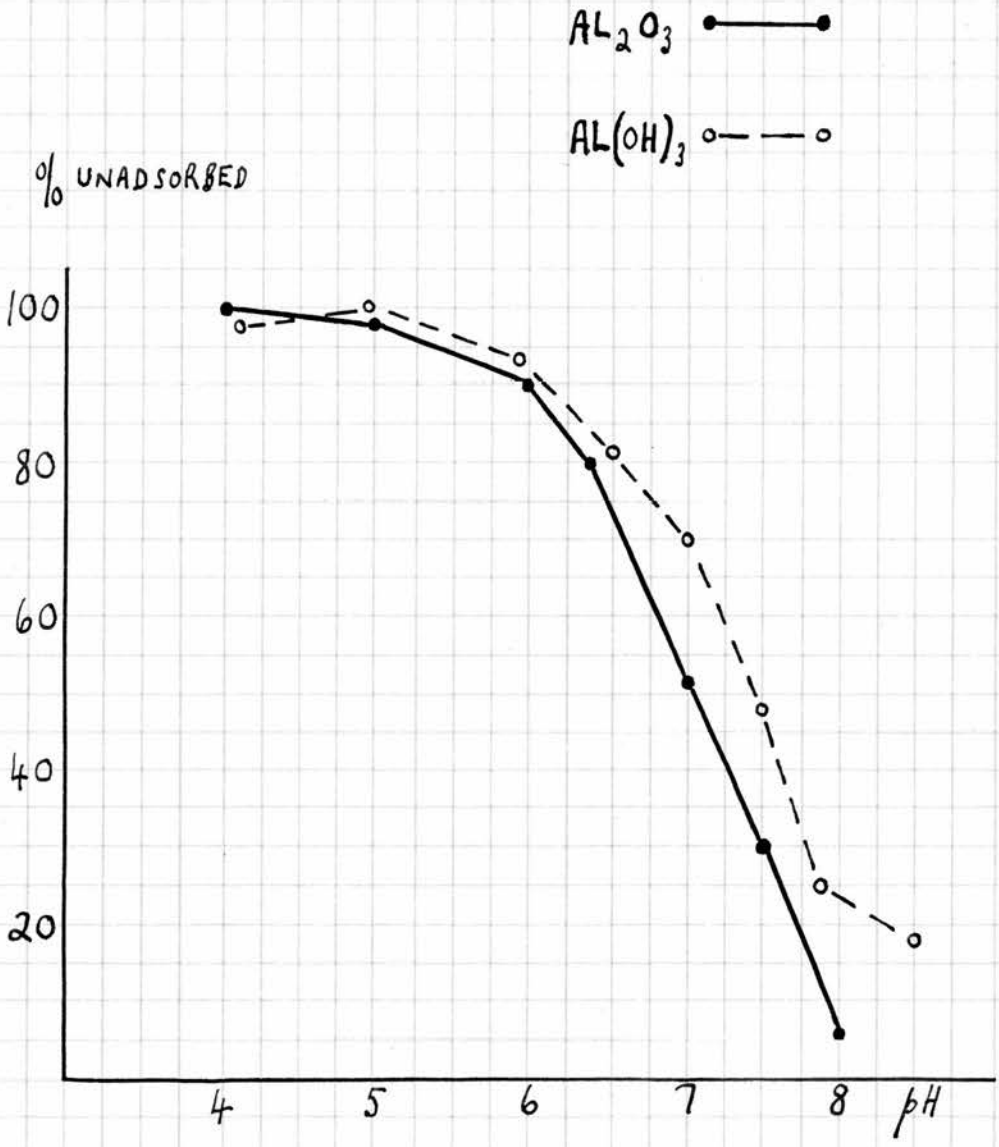
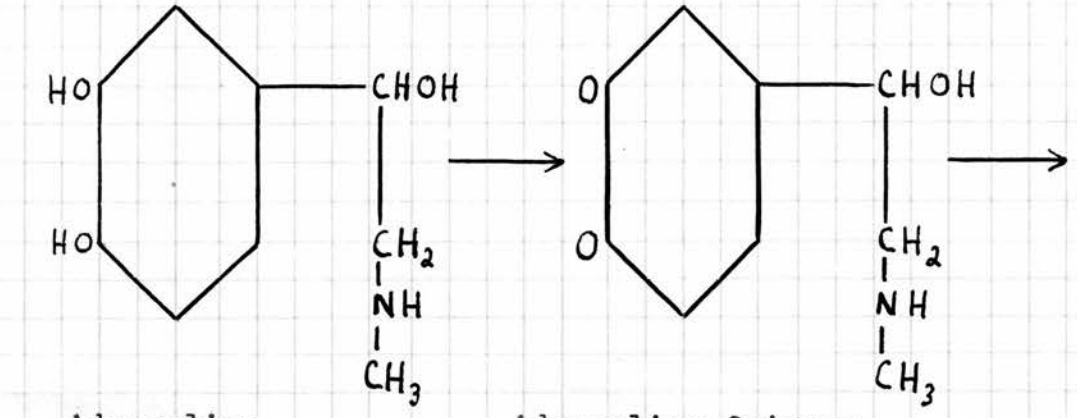


Figure 12.

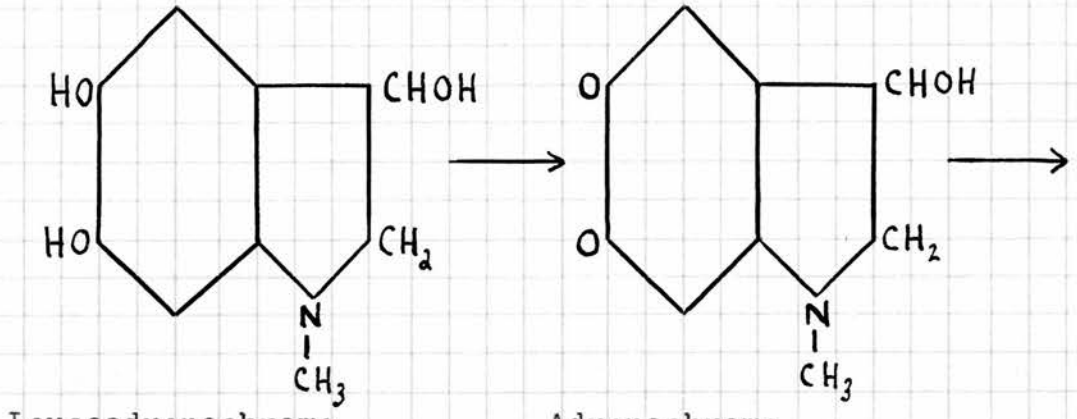
Adsorption of adrenaline on aluminium hydroxide and oxide.

Lund (1949)

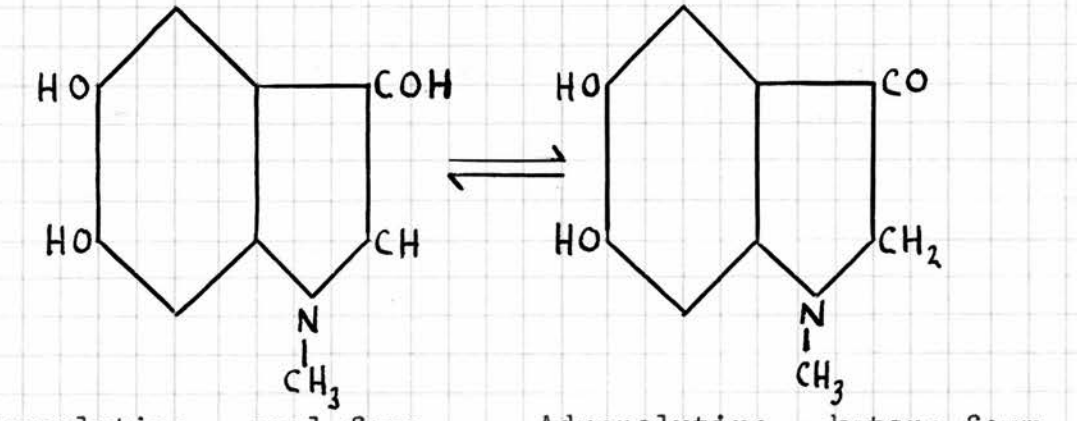
Figure 13.



Adrenaline — Adrenaline Quinone



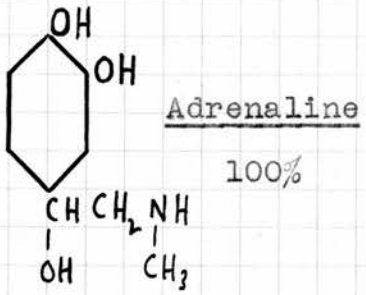
Leucoadrenochrome — Adrenochrome



Adrenolutine - enol form — Adrenolutine - ketone form

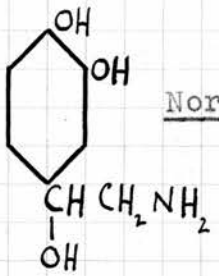
Lund (1949b)

Figure 14.



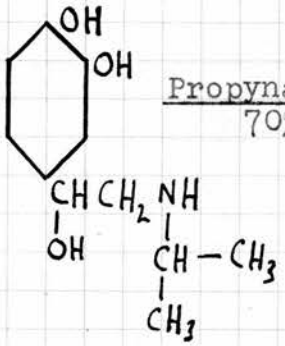
Adrenaline

100%



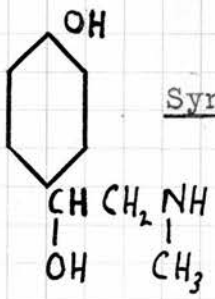
Noradrenaline

10%

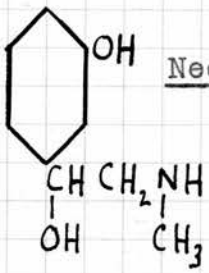


Propynaline

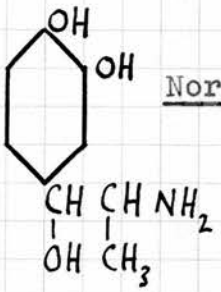
70%



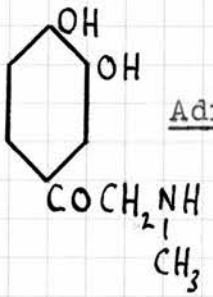
Synephrine



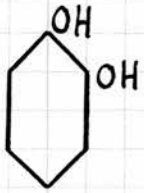
Neosynephrine



Norephrine



Adrenalone



Catechol

Lund (1949c)

Figure 15.

Ethylene diamine condensation reaction
(Natelson et al. 1949)

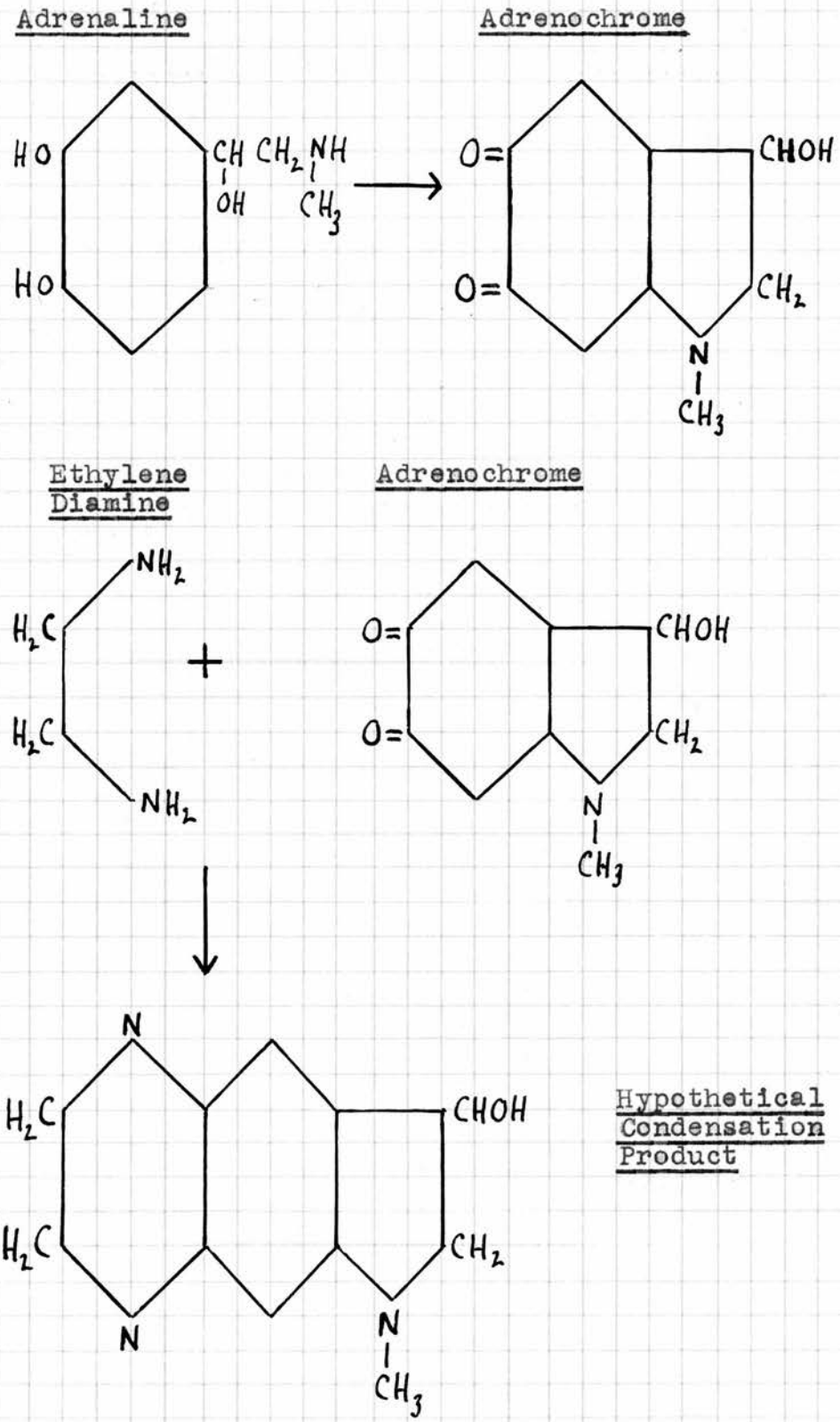
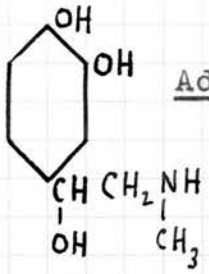
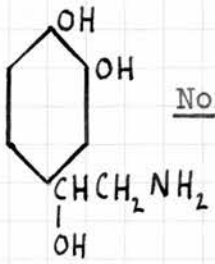
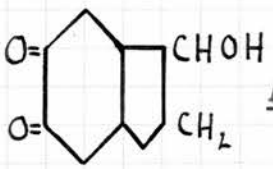
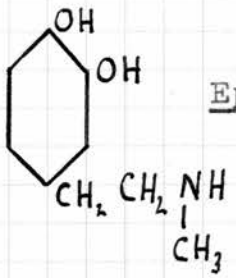
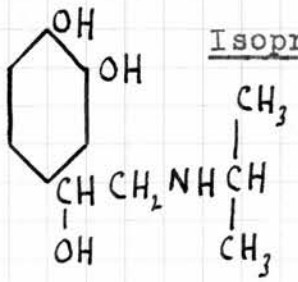
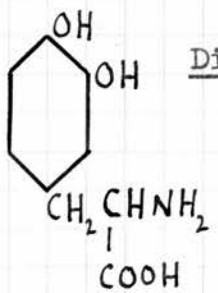
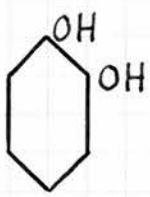
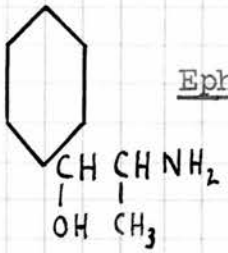
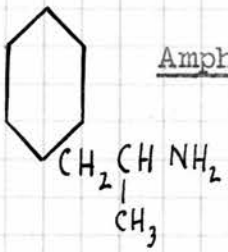
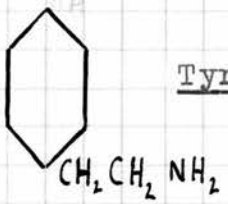
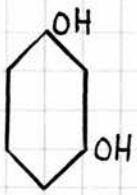
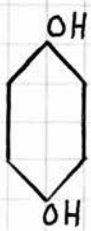
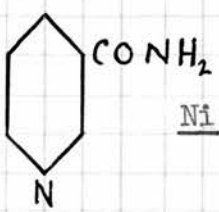


Figure 16.

Catechol Derivative	Relative Fluorescence
 Adrenaline	100
 Noradrenaline	20
 Adrenochrome	100
 Epinine	48
 Isoprenaline	56
 Dihydroxyphenylalanine	0·2
 Catechol	96

Weil-Malherbe and Bone (1952a)

Figure 16 (cont'd.)

Other Compounds	Relative Fluorescence
 Ephedrine	2×10^{-4}
 Amphetamine	1×10^{-6}
 Tyramine	3×10^{-3}
 Resorcinol	0
 Quinol	0
 Nicotinamide	0

Weil-Malherbe and Bone (1952a)

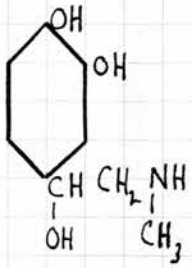
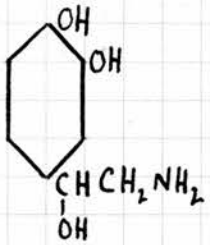
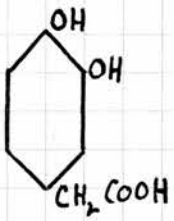
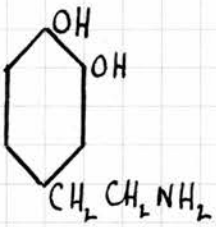
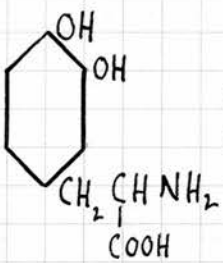
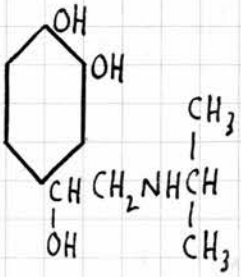
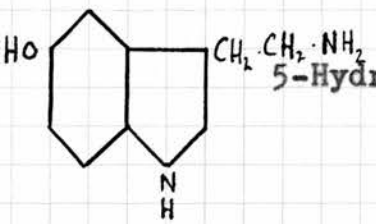
TABLE II.

ADRENALINE				NORADRENALINE			
ug. added	No. of samples	% recovered	S.D.	ug. added	No. of samples	% recovered	S.D.
0.001	4	45	56%	0.01	10	83	49%
0.003	9	69	22%	0.02	6	72	14%
0.006	7	78	13%	0.04	14	73	17%

Recovery of adrenaline and noradrenaline added to plasma

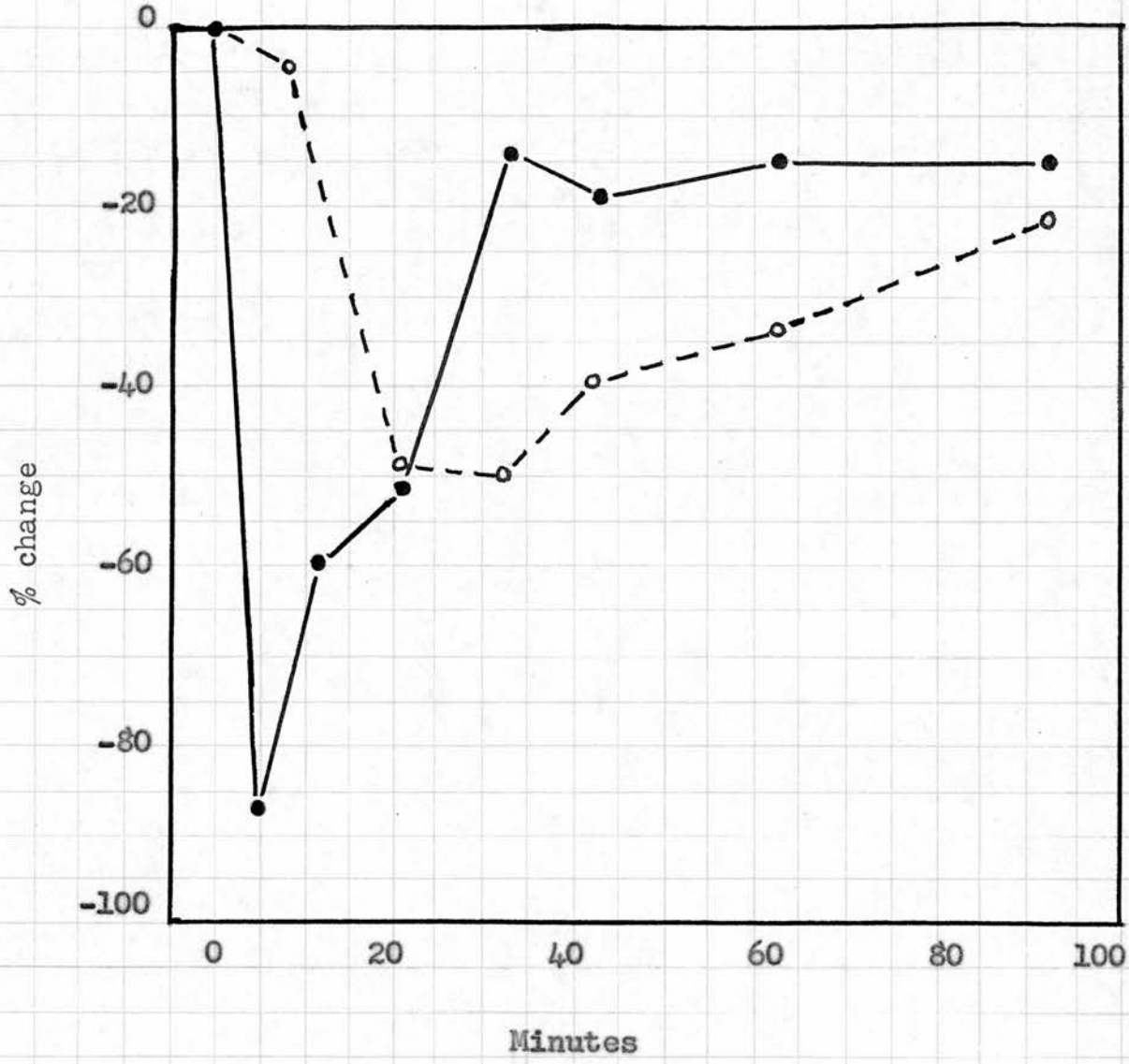
Valk and Millar (1955)

Figure 17.

Dihydroxyphenol Compounds	µg. added	% recovery	Fluorescence ratio 500/580 mµ
 Adrenaline	.001-0.20	78	0.58
 Noradrenaline	0.005-4.0	80	3.74
 3:4-dihydroxyphenyl acetic acid	0.08-0.40	65	4.04
 Hydroxytyramine	0.02-50	79	1.13
 Dihydroxyphenyl-alanine	50	94	1.69
 Isopropyl-noradrenaline	0.25-0.50	72	0.73
 5-Hydroxytryptamine	150-250	0.17	2.22

Valk (1956)

Figure 18.



●——● % change in adrenaline conc.
Initial conc. 1.56 µg./litre of blood.

o- - - -o % change in glucose conc.
Initial conc. 75.5 mgm.%.

Weil-Malherbe (1953)

STUDY 1.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	10	21	67
b	10	20	68.5
2a	10	17	51
b	10	16	49
3a	8.5	20	56
b	8.5	22.5	69
4a	10	22	56
b	10	23	58
5a	10	18	54
b	10	17	50
6a	10	16	47
b	10	16	45
Reagent blank		13.5	41.75
4 A		47.25	60
4 N		22.5	66.5

580 $8.4375\ A + 2.25\ N =$

500 $4.5625\ A + 6.1875\ N =$ (Factor = 2.75)

580 $23.2031\ A + 6.1875\ N =$

$\therefore\ 18.6406\ A = (580 \times 2.750) - 500.$

N.B. Before construction of equations the reagent blank fluorescence values are subtracted from the fluorescence readings of adrenaline (4A) and noradrenaline (4N) standard solutions.

STUDY 1.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (Corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	7.5	25.25	-0.2481	-0.36
b	6.5	26.75	-0.4761	
2a	3.5	9.25	0.0201	0
b	2.5	7.25	-0.0201	
3a	6.5	14.25	0.2288	0.04
b	9.0	27.25	-0.1578	
4a	8.5	14.25	0.4895	0.51
b	9.5	16.25	0.5297	
5a	4.5	12.25	0.0067	0.04
b	3.5	8.25	0.0738	
6a	2.5	5.25	0.0872	0.14
b	2.5	3.25	0.1945	

STUDY 2

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	9	18	55
b	9	15.5	50
2a	9	23.5	62
3a	9	31.8	65
3b	9	26	55
4a	9	27	62.5
b	9	26	62
5a	9	26.5	70
b	9	21	58
6a	9	19.5	55
b	9	21	60.5
7a	8	16.5	52
b	8	13	43
Reagent blank		12.25	39.25
4 A		44.5	61.75
4 N		23.25	72.5

580 8.0625 A + 2.75 N =

500 5.625 A + 8.3125 N = (Factor = 3.023)

580 24.3729 A + 8.3125 N =

$\therefore$ 18.748 A = (500 x 3.023) - 580.

STUDY 2.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (Corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	5.75	15.75	0.0968	0.02
b	3.25	10.75	-0.0549	
2a *	11.25	22.75	0.6672	0.67
3a	19.5	25.75	1.9674	1.75
b	13.75	15.75	1.5300	
4a	14.75	23.25	1.2647	1.19
b	13.75	22.75	1.1151	
5a	14.25	30.75	0.7307	0.59
b	8.75	18.75	0.4564	
6a	7.25	15.75	0.3654	0.34
b	8.75	21.25	0.3082	
7a	4.25	12.75	0.0065	-0.05
b	0.75	3.75	-0.0989	

* Plasma volume insufficient for duplicate sample.

STUDY 3.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	8	16	46
b	8	15.5	44.5
2a	9	17.5	46
b	10	19	50
3a	9	26	53.5
b	8	31	63.5
4a	10	30	57
b	8	27	56.5
5a	10	20	49
b	9	19	47.5
6a	10	19	50
b	9	17	46
Reagent blank		12.72	38.41
4 A		39.5	55.75
4 N		22.5	61.5

580 $6.695\ A + 2.445\ N =$

500 $4.335\ A + 5.7725\ N =$ (Factor = 2.361)

580 $15.8069\ A + 5.7725\ N =$

$\therefore\ 11.472\ A = (580 \times 2.361) - 500.$

STUDY 3.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (Corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	3.28	7.59	0.0168	0.03
b	2.78	6.09	0.0516	
2a	4.78	7.59	0.3579	0.32
b	6.28	11.59	0.2909	
3a	13.28	15.09	1.5752	1.77
b	18.28	25.09	1.9689	
4a	17.28	18.59	1.9359	1.82
b	14.28	18.09	1.7025	
5a	7.28	10.59	0.5752	0.57
b	6.28	9.09	0.5557	
6a	6.28	11.59	0.2822	0.26
b	4.28	7.59	0.2436	

STUDY 4.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	8	23.5	71
b	8	21.5	67
2a	8	27.5	74
b	7.5	27	70.5
3a	9	32.5	86
b	9	28.5	71
4a	8	20.5	55.5
b	8	21	55.5
5a	9	20.5	64
b	9	19	60
Reagent blank		16	46.75
4 A		45	61.5
4 N		24.25	70.5

580 $\quad 7.250\ A + 2.0625\ N =$

500 $\quad 3.6875\ A + 5.9365\ N =$ (Factor = 2.879)

580 $\quad 20.87275\ A + 5.9375\ N =$

$\therefore \quad 17.185\ A = (580 \times 2.879) - 500.$

STUDY 4.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (Corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	7.5	24.25	-0.1933	-0.26
b	5.5	20.25	-0.3213	
2a	11.5	27.25	0.4261	0.52
b	11	23.75	0.6144	
3a	16.5	39.25	0.5337	0.65
b	12.5	24.25	0.7589	
4a	4.5	8.75	0.3059	0.36
b	5.0	8.75	0.4106	
5a	4.5	17.25	-0.2777	-0.29
b	3	13.25	-0.2983	

STUDY 5.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	7	17	59
b	7	20	57
2a	5.5	16	46
b	5	21	57
3a	8	43.5	73
b	7	40	69
4a	8.5	36.5	71
b	8	41	87
5	9	21	62
Reagent blank		13	41.25
4 A		44.75	63
4 N		27.5	85

580 $7.9375\ A + 3.625\ N =$

500 $5.4375\ A + 10.9375\ N =$ (Factor = 3.017)

580 $23.9474\ A + 10.9375\ N =$

$\therefore\ 18.510\ A = (580 \times 3.017) - 500.$

STUDY 5.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (Corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	4	17.75	-0.4386	-0.01
b	7	15.75	0.4144	
2a	3	4.75	0.4226	0.66
b	8	15.75	0.9062	
3a	30.5	31.75	4.0699	4.11
b	27	27.75	4.1451	
4a	23.5	29.75	2.6154	2.62
b	28	45.75	2.6153	
5*	8	20.75	0.2032	0.20

* Insufficient plasma volume for duplicate sample.

STUDY 6.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	9	17.5	53
b	9	16	50
2a	9	25	57
b	8	25	58
3a	8	30.5	60.1
b	8	37	80
4a	10	35	64.5
b	9	34	64
5a	9	18.5	52.5
b	9	18	50.5
Reagent blank		13.5	41.25
4 A		32.8	56
4 N		22.25	70

580 4.825 A + 2.188 N =

500 3.688 A + 7.188 N = (Factor = 3.285)

580 15.850 A + 7.188 N =

$\therefore$ 12.162 A = (580 x 3.285) - 500.

STUDY 6.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (Corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	4	11.75	0.1270	0.04
b	2.5	8.75	-0.0491	
2a	11.5	15.75	2.0124	2.09
b	11.5	16.75	2.1613	
3a	17	18.85	3.8024	3.88
b	23.5	38.75	3.9516	
4a	21.5	23.25	3.8955	3.99
b	20.5	22.75	4.0740	
5a	5	11.25	0.4727	0.49
b	4.5	9.25	0.5054	

STUDY 7.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	7	16.0	51.5
b	7	17.5	56
2a	8	32.6	82.3
b	8	29.9	78
3a	8	26.9	59
b	8	25.8	58.3
4a	8	31	63.5
b	7.5	32.2	68.5
5a	8	18.2	53.7
b	8	21	60
6a	8	19.2	58
b	8	16.5	50.6
Reagent blank		10.6	33.85
4 A		50.9	59.25
4 N		20.4	67.5

580 10.075 A + 2.45 N =

500 6.35 A + 8.4125 N = (Factor = 3.434)

580 34.59755 A + 8.4125 N =

$\therefore$ 28.248 A = (580 x 3.434) - 500.

STUDY 7.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	5.4	17.65	0.0451	0.06
b	6.9	22.15	0.0781	
2a	22	48.45	1.1991	1.09
b	19.3	44.15	0.9791	
3a	16.3	25.15	1.3640	1.30
b	15.2	24.45	1.2279	
4a	20.4	29.65	1.7880	1.83
b	21.6	34.65	1.8656	
5a	7.6	19.85	0.2765	0.35
b	10.4	26.15	0.4233	
6a	8.6	24.15	0.2381	0.20
b	5.9	16.75	0.1554	

STUDY 8.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	10	18	50.5
b	8	34	105
2a	10	18.5	51
b	9	19	52
3a	10	30.5	59.5
b	9	28.5	56
4a	10	25	50
b	9	25	50.5
5a	10	31.5	60.1
b	10	35	71
6a	10	20	51.5
b	9	18.5	47.5
Reagent blank		14.75	40.5
4 A		37.25	55
4 N		23	65.25

580 5.625 A + 2.0625 N =

500 3.625 A + 6.1875 N = (Factor = 3.00)

580 16.875 A + 6.1875 N =

$\therefore$ 13.250 A = (580 x 3.0) - 500.

STUDY 8.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	3.25	10	-0.0189	-0.33
b	19.25	64.5	-0.6368	
2a	3.75	10.5	0.0566	0.08
b	4.25	11.5	0.1058	
3a	15.75	19.0	2.1321	2.15
b	13.75	15.5	2.1593	
4a	10.25	9.5	1.6038	1.67
b	10.25	10	1.7400	
5a	16.75	19.6	2.3132	2.30
b	20.25	30.5	2.2830	
6a	5.25	11.0	0.3585	0.36
b	3.75	7.0	0.3564	

STUDY 9.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	8	12.5	42.5
b	7	14	48
2a	8	17	47
b	7	18	50
3a	7	17.5	48
b	7	18.5	51
4a	8	19	49
b	7	20	51.5
5a	8	22.5	56.5
b	7	20	53.5
6a	8	16	52
b	8	14	46
Reagent blank		10.25	33.25
4 A		36.5	51.75
4 N		19.75	64

580 $\quad 6.5625\ A + 2.375\ N =$

500 $\quad 4.625\ A + 7.6875\ N =$ (Factor = 3.237)

580 $\quad 21.242\ 8\ A + 7.6875\ N =$

$\therefore \quad 16.618\ A = (580 \times 3.237) - 500.$

STUDY 9.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	2.25	9.25	-0.1480	-0.19
b	3.75	14.75	-0.2244	
2a	6.75	13.75	0.6093	0.66
b	7.75	16.75	0.7167	
3a	7.25	14.75	0.7494	0.76
b	8.25	17.75	0.7699	
4a	8.75	15.75	0.9358	1.05
b	9.75	18.25	1.1443	
5a	12.25	23.25	1.2339	1.10
b	9.75	20.25	0.9723	
6a	5.75	18.75	-0.0104	-0.03
b	3.75	12.75	-0.0460	

Study 10.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	8	17.5	52.5
b	8	15.5	47.5
2a	8	17	47.5
b	8	17	47
3a	8	19	50.5
b	8	20	54.5
4a	8	22	57.5
b	8	20.2	53
5a	8	19	50.5
b	8	19.5	52
6a	8	26	83
b	8	17.5	53
Reagent blank		14.5	41.4
4 A		50.5	59.9
4 N		27.1	81.5

580 $\quad 9.0\ A + 3.15\ N =$

500 $\quad 4.625\ A + 10.025\ N =$ $\quad$ (Factor = 3.183)

580 $\quad 28.647\ A + 10.025\ N =$

$\therefore \quad 24.022\ A = (580 \times 3.183) - 500.$

STUDY 10.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (corrected for plasma μg/litre	Mean of the duplicate samples μg/litre
1a	3.0	11.1	-0.0813	-0.12
b	1.0	6.1	-0.1518	
2a	2.5	6.1	0.0966	0.11
b	2.5	5.6	0.1228	
3a	4.5	9.1	0.2718	0.25
b	5.5	13.1	0.2293	
4a	7.5	16.1	0.4045	0.37
b	5.7	11.6	0.3405	
5a	4.5	9.1	0.2718	0.27
b	5.0	10.6	0.2766	
6a	11.5	41.6	-0.2599	-0.18
b	3.0	11.6	-0.1068	

STUDY 11.

Sample No.	Volume (ml.)	Fluorescence 580 mμ	Fluorescence 500 mμ
1a	7	16	48.5
b	8	16	48.5
2a	7	20	53
b	8	19	49.5
3a	7	19.5	51
b	8	23	61
4a	7	19.5	50
b	8	21	52
5a	7	20	51
b	8	21	52.5
6a	7	17	49
b	8	17	48.5
Reagent blank		13.75	39.5
4 A		49.25	60.75
4 N		25.75	76.75

580 8.875 A + 3.00 N =

500 5.3125 A + 9.3125 N = (Factor = 3.104)

580 27.548 A + 9.3125 N =

$\therefore$ 22.236 A = (580 x 3.104) - 500.

STUDY 11.

Sample No.	Net fluorescence after subtraction of blank values 580 mμ	500 mμ	Estimated adrenaline (corrected for plasma volume) μg/litre	Mean of the duplicate samples μg/litre
1a	2.25	9.0	-0.1296	-0.12
b	2.25	9.0	-0.1134	
2a	6.25	13.5	0.3790	0.37
b	5.25	10.0	0.3539	
3a	5.75	11.5	0.4079	0.41
b	9.25	21.5	0.4054	
4a	5.75	10.5	0.4720	0.52
b	7.25	12.5	0.5624	
5a	6.25	11.5	0.5076	0.52
b	7.25	13.0	0.5343	
6a	3.25	9.5	-0.0377	-0.05
b	3.25	9.0	-0.0611	

TABLE III ADRENALINE

Study No.	Source of variation	DF	Sum of squares	Mean square	F	P
1	Total	11	0.8932			
	Between samples	5	0.7829	.1566	8.51	< 0.02
	Within samples	6	0.1104	.0184		
2	Total	12	5.1234			
	Between samples	6	4.7376	.7896	12.28	< 0.01
	Within samples	6	0.3850	.0643		
3	Total	11	6.3879			
	Between samples	5	6.2794	1.2559	69.44	< 0.01
	Within samples	6	0.1085	.0181		
4	Total	9	1.6049			
	Between samples	4	1.5479	.3870	33.96	< 0.01
	Within samples	5	0.0570	.0114		
5	Total	8	23.9860			
	Between samples	4	23.4818	5.8704	46.57	< 0.01
	Within samples	4	0.5042	.1261		
6	Total	9	27.1605			
	Between samples	4	27.1063	6.7766	625.29	< 0.01
	Within samples	5	0.0542	.0108		
7	Total	11	5.0423			
	Between samples	5	4.9911	.9982	116.95	< 0.01
	Within samples	6	0.0512	.0085		
8	Total	11	13.1239			
	Between samples	5	12.9217	2.5843	76.68	< 0.01
	Within samples	6	.2022	.0337		
9	Total	11	3.0304			
	Between samples	5	2.9670	0.5934	56.12	< 0.01
	Within samples	6	.0635	0.0106		
10	Total	11	.5228			
	Between samples	5	.5053	.1011	34.63	< 0.01
	Within samples	6	.0175	.0029		
11	Total	11	.8197			
	Between samples	5	.8146	0.1629	189.23	< 0.01
	Within samples	6	.0052	0.0009		
All Studies.	Within samples	62	1.5595	.0252	-	-

TABLE IV. ADRENALINE

Study No.	S.E.	M.S.D.
1	0.1356	0.33
2	0.2536	0.62
3	0.1345	0.33
4	0.1067	0.27
5	0.3550	0.99
6	0.1041	0.27
7	0.0924	0.23
8	0.1836	0.45
9	0.1028	0.25
10	0.0540	0.13
11	0.0293	0.07
All studies	0.1586	0.32

TABLE V.

Study No.	Sample No. 1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b	7a	7b
1	4.26	4.67	1.48	1.19	2.54	5.30	1.94	2.24	1.98	1.28	0.78	0.38		
2	2.04	1.47	2.59		2.11	1.07	2.25	2.29	3.62	2.20	1.86	2.63	1.91	0.63
3	1.63	1.28	1.19	1.77	1.72	3.95	1.77	2.64	1.40	1.33	1.80	1.28		
4	5.22	4.46	5.47	4.95	7.01	4.07	1.65	1.59	1.45	0.57				
5	2.54	1.85	0.58	2.43	1.61	1.56	1.90	3.93	2.01					
6	1.75	1.38	1.40	1.80	1.33	4.71	1.24	1.43	1.50	1.17				
7	2.96	3.70	6.29	5.82	2.71	2.71	3.06	4.12	2.74	3.57	3.41	2.37		
8	1.63	13.40	1.66	2.00	1.82	1.52	0.60	0.78	1.81	3.59	1.57	1.05		
9	1.59	2.88	1.87	2.68	2.29	2.84	1.99	2.70	3.04	3.18	3.06	2.10		
10	1.42	0.83	0.72	0.64	1.01	1.53	1.82	1.29	1.01	1.19	6.06	1.50		
11	1.45	1.27	1.86	1.14	1.53	2.66	1.34	1.36	1.47	1.44	1.66	1.54		

Noradrenaline values, insulin studies.

TABLE VI NORADRENALINE

Study No.	Source of variation	DF	Sum of Squares	Mean Square	F	P
1	Total	11	27.6673			
	Between samples	5	23.3724	4.6745	6.52	<0.05
	Within samples	6	4.3049	.7175		
2	Total	12	6.5856			
	Between samples	6	0.4037	0.0673	0.07	
	Within samples	6	6.1819	1.0303		
3	Total	11	6.6569			
	Between samples	5	3.4249	.6850	1.27	
	Within samples	6	3.2320	.5387		
4	Total	9	42.1590			
	Between samples	4	37.0242	9.2561	9.01	<0.01
	Within samples	5	5.1348	1.0270		
5	Total	8	6.5774			
	Between samples	4	.5464	.1366	0.09	
	Within samples	4	6.0310	1.5078		
6	Total	9	9.9569			
	Between samples	4	4.0238	1.0060	0.85	
	Within samples	5	5.9331	1.1866		
7	Total	11	17.0142			
	Between samples	5	15.1829	3.0366	9.95	<0.01
	Within samples	6	1.8313	.3052		
8	Total	11	132.9749			
	Between samples	5	61.8701	12.3740	1.04	
	Within samples	6	71.1048	11.8508		
9	Total	11	3.0852			
	Between samples	5	1.0512	.2102	0.62	
	Within samples	6	2.0340	.3390		
10	Total	11	23.2339			
	Between samples	5	12.3680	2.4736	1.37	
	Within samples	6	10.8659	1.8110		
11	Total	11	1.6948			
	Between samples	5	.7731	.1546	1.01	
	Within samples	6	.9217	.1536		

TABLE VII NORADRENALINE

Study No.	S.E.	M.S.D.
1	± 0.85	2.07
2	± 1.02	2.48
3	± 0.73	1.80
4	± 1.01	2.60
5	± 1.23	3.41
6	± 1.09	2.80
7	± 0.55	1.35
8	± 3.44	8.43
9	± 0.58	1.42
10	± 1.35	3.29
11	± 0.39	0.96

TABLE VIII

TIME RELATIVE TO INSULIN	HEART RATE Ballisto.	ARTERIAL BLOOD PRESSURE	ESTIMATED CARDIAC STROKE VOLUME	ESTIMATED CARDIAC OUTPUT	ESTIMATED ADRENALINE per litre
-5	76	115/60	71 cc.	5.4 litres	-0.19 µg.
+27	100	138/64	80 cc.	8.0 litres	0.66 µg.
+80	80	130/50	89 cc.	7.1 litres	1.10 µg.
+128	56	100/60	74 cc.	4.1 litres	-0.03 µg

Subject 3

TIME RELATIVE TO INSULIN	HEART RATE Ballisto.	ARTERIAL BLOOD PRESSURE	ESTIMATED CARDIAC STROKE VOLUME	ESTIMATED CARDIAC OUTPUT	ESTIMATED ADRENALINE per litre
-2	60	105/55	66 cc.	4.0 litres	-0.12 µg.
+45	59	115/50	80 cc.	4.7 litres	0.25 µg.
+65	59	120/55	90 cc.	5.3 litres	0.37 µg.
+115	57	100/70	78 cc.	4.5 litres	-0.18 µg.

Subject 4

TIME RELATIVE TO INSULIN	HEART RATE Ballisto.	ARTERIAL BLOOD PRESSURE	ESTIMATED CARDIAC STROKE VOLUME	ESTIMATED CARDIAC OUTPUT	ESTIMATED ADRENALINE per litre
-2	84	105/65	64 cc.	5.4 litres	-0.12 µg
+75	90	125/60	90 cc.	8.1 litres	0.41 µg.
+125	84	115/70	77 cc.	6.5 litres	-0.05 µg

Subject 5

INSULIN HYPOGLYCAEMIA

Infusion 1.

Sample No.	Plasma Volume (ml.)	Fluorescence 580 mμ.	Fluorescence 500 mμ.
1a	8	26.8	62.4
b	8	28.0	63.0
2a	8	25.9	64.9
b	8	23.3	56.2
3a	8	25.7	56.5
b	8	26.8	59.2
4a	8	35.3	68.2
b	8	35.2	66.9
5a	9	31.8	86.1
b	10	29.8	70.6
Reagent blank		21	49.5
4 A		55.75	65.0
4 N		35	91.75

580 8.6875A+ 3.5 N =

500 3.875A + 10.5625N =

580 26.21888A + 10.5625 N =

$\therefore$ 22.344A = 580 x 3.018 - 500

Infusion I.

Sample No.	Net Fluorescence after subtraction of blank values 580 mμ.	500 mμ.	Estimated Adrenaline (Corrected for plasma volume)	Mean of the duplicate samples
1a	5.8	12.9	0.2576	0.34
b	7.0	13.5	0.4266	-
2a	4.9	15.4	-0.0343	-0.01
b	2.3	6.7	0.0135	-
3a	4.7	7.0	0.4020	0.42
b	5.8	9.7	0.4366	-
4a	14.3	18.7	1.3683	1.40
b	14.2	17.4	1.4241	-
5a	10.8	36.6	-0.1989	0.02
b	8.8	21.1	0.2443	-

Infusion 2.

Sample No.	Plasma volume (ml.)	Fluorescence 580 mu.	500 mu.
1a	10	25.0	50.0
b	10	20.9	39.1
2a	10	22.1	43.0
b	10	21.8	42.4
3a	10	19.9	39.0
b	9	18.2	37.0
4a	10	21.7	39.3
b	10	21.0	38.0
5a	10	28.8	66.0
b	10	20.4	41.5
	Reagent blank	16.5	31
	4 A	46.5	46.75
	4 N	28.125	57.125

580 $7.5\ A + 2.90625\ N =$

500 $3.9375\ A + 6.53125\ N =$

580 $16.8525\ A + 6.53125\ N =$

$12.915\ A = 580 \times 2.247 - 500$

Infusion 2.

Sample No.	Net Fluorescence after subtraction of blank values 580 mu	500 mu.	Estimated Adrenaline (Corrected for plasma volume)	Mean of the duplicate samples
1a	8.5	19	.0077	0.07
b	4.4	8.1	0.1383	
2a	5.6	12	0.0452	0.04
b	5.3	11.4	0.0394	
3a	3.4	8.0	-0.0279	-0.11
b	1.7	6.0	-0.1876	
4a	5.2	8.3	0.2621	0.25
b	4.5	7.0	0.2409	
5a	12.3	35	-0.5700	-0.33
b	3.9	10.5	-0.0825	

TABLE IX

INFUSION	TIME OF INFUSION	HEART RATE	ARTERIAL BLOOD PRESSURE	ESTIMATED CARDIAC STROKE VOLUME	ESTIMATED CARDIAC OUTPUT	ESTIMATED ADRENALINE per litre
CONTROL	15 mins.	64	105/70	57 cc.	3·7 litres.	——
3 µg./minute	23 mins.	76	120/55	63 cc.	4·8 litres.	0·34 µg.
CONTROL	34 mins.	70	110/70	57 cc.	4·0 litres.	−0·01 µg.
2 µg./minute	15 mins.	69	108/66	62 cc.	4·3 litres.	0·42 µg.
6 µg./minute	12 mins.	76	136/62	64 cc.	4·9 litres.	1·40 µg.
CONTROL	42 mins.	67	115/80.	57 cc.	3·8 litres.	0·02 µg.

Subject 3

Infusion of L-ADRENALINE 2 µg./ml.

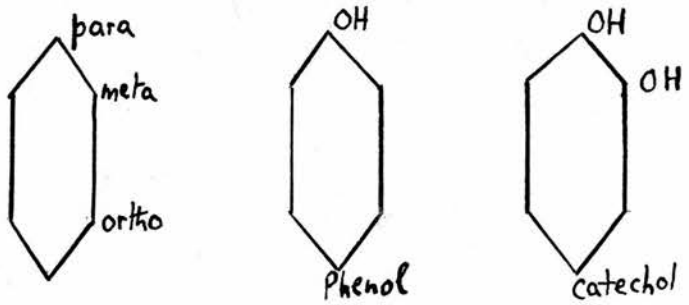
TABLE X

INFUSION	TIME OF INFUSION	HEART RATE	ARTERIAL BLOOD PRESSURE	ESTIMATED CARDIAC STROKE VOLUME	ESTIMATED CARDIAC OUTPUT	ESTIMATED ADRENALINE per litre.
CONTROL	10 mins.	68	112/63	65 cc.	4.4 litres	0.07 µg.
1 µg./minute	33 mins.	78	112/53	65 cc.	5.1 litres.	0.04 µg.
CONTROL	22 mins.	72	108/65	58 cc.	4.2 litres	−0.11 µg.
3 µg./minute	32 mins.	80	130/55	66 cc.	5.3 litres	0.25 µg.
CONTROL	23 mins.	70	115/70	58 cc.	4.1 litres	−0.33 µg.

Subject 4

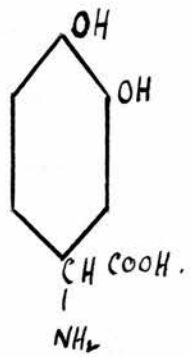
Infusion of L-ADRENALINE 1 µg./ml.

1. Benzene Ring - This is a hexagon containing six carbon atoms to each of which a hydrogen atom is attached. Substitution of one hydroxyl group produces hydroxybenzene (phenol); introduction of two hydroxyl groups gives dihydroxybenzene (hydroxyphenol catechol).



2. Epinine and Noradrenaline - Page 105.
Dihydroxyphenylethylamine - hydroxytyramine, Page 108.

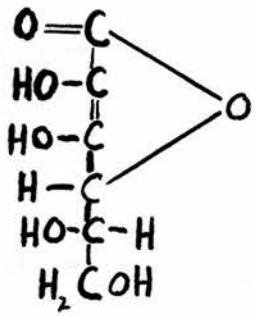
3. Amino-aceto catechol



4. Aromatic polyhydroxy compounds - Derivatives of benzene (C_6H_6), containing more than one hydroxyl group.

5. pH = -log. hydrogen ion concentration = log. $\frac{1}{H^+}$ indicating the inverse relationship between H^+ and OH^- in solutions of acids and bases.

6. Ascorbic acid - Vitamin C, one of the group of substances known as enediols, which contain two adjacent carbon atoms linked by a double bond and substituted with an OH group at each carbon atom.



7. Basic dissociation constant (K) - see Note 9.

8. Alkalylamines - amines (i.e. containing the group NH_2, substituted or unsubstituted) derived from hydrocarbons of the general formula C_nH_{2n+1}.

9. Law of Mass Action - The rate at which a substance reacts is proportional to its active mass (molecular weight expressed in gms.) and hence the velocity of a chemical reaction is proportional to the products of the molecular concentrations of the reactants. Thus, in the reaction $A + B \rightleftharpoons C + D$, the velocity of the forward reaction is $V = Vf = k_1[A][B]$. The velocity of the backward reaction is $V_b = k_2[C][D]$.

At equilibrium $Vf = Vb$

or $k_1[A][B] = k_2[C][D]$.

hence $\frac{k_1}{k_2} = \frac{[C][D]}{[A][B]} = K$, the Dissociation Constant.

10. Non-basic polyhydroxy phenol compounds - Non-basic compounds in which several hydrogen atoms of the benzene nucleus have been replaced by hydroxyl groups.

11. Triose - A carbohydrate (monosaccharide) containing 3 carbon atoms. 3 trioses are known - dihydroxyacetone and 2 stereoisomers of glyceraldehyde.

Diose - A carbohydrate (monosaccharide) containing 2 carbon atoms.

12. Pentoses and hexoses - Carbohydrates containing 5 and 6 carbon atoms respectively.

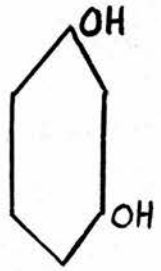
13. Ergothioneine - A nitrogenous base, containing sulphur and present in blood; ergothioneine is found in ergot.

14 (a) Thiols - Hydrosulphide compounds of the type R - SH i.e., containing the monovalent + radical SH.

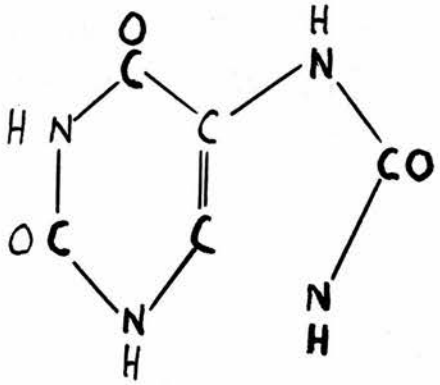
(b) Enediols - Substances containing 2 adjacent carbon atoms linked by a double bond and substituted with an OH group at each carbon atom, e.g. ascorbic acid (Note 6).

15 (a) Monohydric phenols - One hydrogen atom in the benzene nucleus is replaced by a hydroxyl group.

(b) Resorcinol - A bactericide, like phenol in action. Resorcinol is widely used in ointments for the treatment of skin conditions.



(c) Uric acid - An end product of protein metabolism.



16. Eserine (physostigmine) - One of several alkaloids isolated from the Calabar bean, important pharmacologically because of its inhibitory activity against cholinesterase, the physiological enzyme responsible for the breakdown of acetyl choline. One of the degradation products of eserine is rubreserine, an orthoquinone related in structure to adrenochrome.

17. 5N. NaOH - A solution containing 5 x 40 gms. sodium hydroxide in one litre. A normal solution (N.) contains one gram-equivalent weight of active substance in 1 litre of solution at 20°C.

18. Dialysis - The basis of this physical process is the separation of a colloidal solution from electrolytes by enclosing the solution in a membrane (e.g. collodion, parchment paper) which is placed in water. Ultrafiltration is a similar process in which a colloidal solution is filtered under pressure through such a membrane, the larger molecules being retained by the filter while the small molecules, together with the solvent, can pass through.

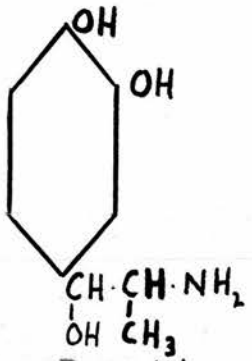
19. Buffer solution - A solution having reserve acidity and alkalinity, taking up considerable amounts of acid or alkali with only slight changes in pH. A buffer is usually defined as a solution containing a weak acid or base with one of its salts.

20. Melanin - The brown pigment derived from tyrosine and produced by oxidation of adrenaline to beyond the stage reached when the fluorescent compound adrenolutine is formed (Page 102).

21. Isomeric - Possessing the property of optical isomerism, i.e. the substance can exist in two forms, dextro and laevo, which rotate the plane of polarised light to right and left respectively. The racemic form of a substance is a mixture containing equal parts of the d- and l- forms.

22. Indoxyl derivatives - Derivatives of 3-hydroxy-indole, present in animal urine.

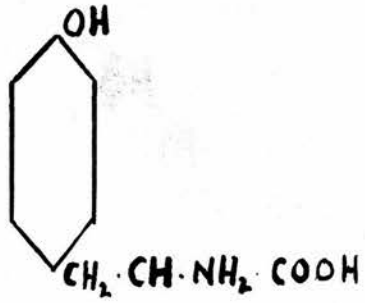
23. Corbasil (cobefrine) - ∝methyl-dl-noradrenaline.



24. Ethylene diamine - Page 104.

25 (a) Tryptophane - One of the essential amino acids, which gives rise to all the derivatives of indole formed during protein putrefaction.

(b) Tyrosine - An amino acid concerned in the synthesis of thyroxine, of adrenaline and noradrenaline, and of the pigment melanin.



26. Rf value - The Rf factor refers to the rate at which a substance (dissolved in a solvent) advances relative to the solvent when a solution spreads over a porous material such as the -cellulose employed for paper chromatography. "By chromatography is meant those processes which allow the resolution of mixtures by effecting separation of some or all of their components in concentration zones on or in phases different from those in which

they are originally present, irrespective of the nature of the force or forces causing the substance to move from one phase to another." (Williams, 1954).

27. In the Farrand photoelectric fluorimeter, the light source is a mercury vapour arc lamp providing ultra-violet and visible radiation. Opening the shutter allows the exciting light beam to pass through a diaphragm with an aperture of variable size. A quartz lens system is used to direct the light in parallel rays through the primary filter on to a glass cuvette with contained sample for estimation. The fluorescent radiation from the sample is collected at right angles to the illuminating beam. By means of a second quartz lens system and secondary filter, the fluorescent beam is used to image the centre of the cuvette on the cathode of a photomultiplier tube. The photoelectrical response is indicated on the attached Rubicon galvanometer.

28. Fick Principle - The general formulas used in the calculation of cardiac output according to the principle outlined by Fick are -

$$\text{Cardiac output(c.c.)} = \frac{CO_2 \text{ output (c.c.)}}{\text{A-V } CO_2 \text{ difference(c.c.\%)}} \times 100$$

or

$$\frac{O_2 \text{ Consumption (c.c.)}}{\text{A-V } O_2 \text{ difference(c.c.\%)}} \times 100$$

The Arterio-venous differences are found by determining the oxygen or carbon dioxide content of arterial blood by arterial puncture, and of mixed venous blood by withdrawing a sample through a catheter passed into the right auricle.

(29) Since the classic paper by Oliver and Schaefer (1895) who described the pressor effects produced by intravenous injection of extracts of adrenal gland, biological methods have been preferred to the less specific chemical techniques for the assay of adrenaline in tissue extracts and body fluids. A detailed analysis of all the methods of biological assay of adrenaline is outside the scope of this presentation; a comprehensive summary of the majority of techniques which have been used for biological assay of adrenaline in blood, commencing with Battelli (1902b), is available elsewhere (Pekkarinen, 1948).

In the course of a recent evaluation, Gaddum, Peart and Vogt (1949) supported the earlier view held by several workers (Schlossmann, 1927; Kahlson and Werz, 1930; Kure, Okinaka, Ohshima, Shimamota and Okamura, 1936) that the perfused rabbit ear was a sensitive preparation for the estimation of adrenaline. For blood analysis, however, the most sensitive preparation was considered to be the rat uterus contracted by acetyl choline (see also Gaddum and Lembeck, 1949).

On the basis of parallel quantitative assays, employing five test preparations (rat uterus, rat colon, rabbit ear, cat spleen cat nictitating membrane) Gaddum, Peart and Vogt (1949) described a method for the estimation of adrenaline and noradrenaline in extracts contain-

ing a mixture of both amines. Application of this technique to blood was adversely affected by interfering substances, however, and the sensitivity of the method, 10 ug. adrenaline, and 100 ug. noradrenaline per litre, precluded its use for the estimation of normal plasma samples.

The isolated rectal caecum of the hen (Bergstrom et al. 1950) and the denervated nictitating membrane of the cat (Bulbring and Burn, 1949) were incorporated into parallel methods of biological assay employed by other workers.

The possibility of accurate biological assay of adrenaline in blood was considerably increased by the use of paper chromatography (James, 1948) for the separation of adrenaline and noradrenaline in plasma samples (Crawford and Outschoorn, 1951). After preliminary extraction of catechol amines by means of acid-ethanol, and their application to paper, the eluates from the adrenaline and noradrenaline spots on the chromatograph paper were assayed separately, using the rat blood pressure. With this technique, assay of 0.25 ug. adrenaline or noradrenaline per ml. could be accomplished, the loss being 0 to 25%.

From subsequent use of this technique of separation of adrenaline and noradrenaline from plasma by paper chromatography, followed by biological assay of

adrenaline on the rat uterus stimulated by carbachol (Vogt, 1952; Holzbauer and Vogt, 1954) it appears that a reliable biological technique is available for the estimation of adrenaline in plasma. The sensitivity of Vogt's technique allows greater than 70% recovery of 0.4 ug. adrenaline per litre. Satisfactory recovery of 1 ug. noradrenaline per litre is obtainable by employing the rat blood pressure. The procedure is a formidable one, however, and its use may tend to be limited to a few special centres where adequate experience and facilities are available.

REFERENCES

ABE, Y. (1924) Arch. exp. Path. Pharmak. 103, 73

ABELOUS, SOULIÉ, TOUJAN (1905) C.R.Soc. Biol. (Paris) 58, 301

ABRAMSON, E. (1933) Skand. Arch. Physiol. 66, 191

AHLGREN, G. (1921) Skand. Arch. Physiol. 41, 1

ALDRICH, T.B. (1901) Amer. J. Physiol. 5, 457

ANGENHEISTER, G., LAU, E. (1928) Naturwissenschaften, 16, 513

ANNERSTEN, S., GRONWALL, A., KÖIW, E. (1949) Nature (Lond.) 163, 136

ARONOW, L., HOWARD, F.A. (1955) Fed. Proc. 14, 315

ARONOW, L., HOWARD, F.A., WOLFF, D. (1956) J.Pharmacol. 116, 1

AUTENRIETH, W., QUANTMEYER, H. (1921) Münch. med. Wschr. 68, 1007

AZZOLINI (1931) Boll. Chim. 70, 665

BAILLY (1924) J.Pharm.Chim., Paris, 30, 404

BAKER, L.C., MARRIAN, G.F. (1927) Biochem.J. 21, 1005

BALL, E.G., CHEN, P.T. (1933) J.Biol.Chem. 102, 703

BANTING, F.G., BEST, C.H. (1922) J.Lab.clin.Med. 7, 251

BANTING, F.G., BEST, C.H., COLLIP, J.B., MACLEOD, J.J.R., NOBLE, E.C. (1922a) Amer.J.Physiol. 62, 162

BANTING, F.G., BEST, C.H., COLLIP, J.B., CAMPBELL, W.R., FLETCHER, A.A. (1922b) Canad.med.Ass.J. 12, 141

BANTING, F.G., CAMPBELL, W.R., FLETCHER, A.A. (1923) Brit. Med.J. 1, 8

BARCROFT, H., STARR, I. (1951) Clin.Sci. 10,295

BARKER, J.H., EASTLAND, C.J., EVERS, N. (1932) Biochem.J. 26, 2129

BATTELLI, F. (1902a) C.R.Soc.Biol. 54, 571

BATTELLI, F. (1902b) C.R.Soc.Biol. 54, 1518

BENDER, M.B., SIEGAL, S. (1939) Amer.J.Physiol. 128, 324

BENNETT, H.S. (1941) Amer.J.Anat. 69, 333

BERGSTROM, S., EULER, U.S. von, HAMBERG, U. (1950) Acta. physiol. Scand. 20, 101

BLOCH, G. (1948) Helv. physiol. pharmacol. Acta, 6, 122.

BLOOR, W.R., BULLEN, S.S. (1941) J.Biol.Chem. 138, 727

BLOTNER, H. (1930) New Eng.J.Med. 203, 709

BOOTHBY, W.M., WILDER, R.M. (1923) Med.Clin.N.Amer. 7, 53

BRANDT, F., KATZ, G. (1933) Z.klin.Med. 123, 23

BRITISH MEDICAL JOURNAL (1956) Annotation, 1, 448

BROSS, W., KUBIKOWSKI, P. (1935) Arch.exp.Path.Pharmak. 178, 212

BULBRING, E., BURN, J.H. (1949) Brit.J.Pharmacol. 4, 202

BU'LOCK, J.D., HARLEY-MASON, J. (1951) J.chem.Soc. 712

BURN, J.H. (1923) J.Physiol. 57, 318

BURN, J.H., HUTCHEON, D.E., PARKER,R.H.O. (1950)Brit.J. Pharmacol. 5, 417

BURN, J.H., MARKS, H.P. (1925) J.Physiol. 60, 131

CANNON, W.B., McIVER, M.A., BLISS, S.W. (1924) Amer. J. Physiol. 69, 46

CEVIDALLI, A. (1909) Arch.Ital.Biol. 52, 59

COLIN, G. (1856) "Comparative Physiology of Domestic Animals," Bailliere & Son, Paris.

COMESSATTI, G. (1909) Berl.klin.Wschr. 46, 356

COURNAND, A., RANGES, H.A., RILEY, R.L. (1942) J.Clin.Invest. 21, 287

CRAWFORD, T.B.B., OUTSCHOORN, A.S. (1951) Brit.J. Pharmacol. 6, 8

CROCETTA, A. (1933) Cited in Chem.Abstr. 27, 5800

DOGLIOTTI,V., CROCETTA, A. (1933) Cited in Chem.Abstr. 27, 5800

DOPY,T., WEISINGER, I. (1938) Z.physiol.Chem. 255, 259

D'SILVA, J.L. (1937) Biochem.J. 31, 2171

DUNER,H. (1953) Acta physiol.Scand. 28 Suppl. 102

EHRLEN,I. (1948) Farm.Revy. 47, 242

EICHLER, O., NOACK,Ch. (1939) Arch.exp.Path.Pharmak. 193, 503

ERNSTENE, A.C., ALTSCHULE, M.D. (1931) J.clin.Invest. 10, 521

ERNSTENE, A.C., RISEMAN,J.E.F., STERN,B., ALEXANDER,B. (1935) Amer.J.Physiol. 111, 440

EULER, U.S. von (1927) Pflügers Arch.ges.Physiol. 217, 699

EULER, U.S. von (1933) Arch.exp.Path.Pharmak. 171, 186

EULER, U.S. von, FLODING, I. (1955) Acta physiol.Scand. 33, Suppl.118

EULER, U.S. von, HAMBERG, U. (1949) Acta physiol.Scand. 19, 74

EULER, U.S. von, HAMBERG, U., HELLNER, S. (1951) Biochem.J. 49, 655

EULER, U.S. von, HOLMQUIST, A.G. (1934) Pflugers Arch. ges. Physiol. 234, 210

EULER, U.S. von, LILJESTRAND, G. (1927) Skand.Arch.Physiol. 52, 243

EULER, U.S. von, LILJESTRAND, G. (1929) Skand.Arch.Physiol. 55, 1

EULER, U.S. von, LUFT, R. (1952) Metabolism 1, 528

EULER, U.S. von, ORWEN, I. (1955) Acta physiol. Scand. 33, Suppl. 118

EULER, U.S. von, SCHMITERLÖW, (1947) Acta physiol. Scand. 13, 1

EWINS, A.J. (1910) J. Physiol. 40, 317

FISCHER, P. (1949) Bull. Soc. chim. Belg. 58, 205

FLETCHER, A.A., CAMPBELL, W.R. (1922) J.Met.Res. 2, 637

FOLIN, O., DENIS, W. (1913) J. Biol. Chem. 13, 469

FOLIN, O., CANNON, W.B., DENIS, W. (1913) J. Biol. Chem. 13, 477

FRÄNKEL, S., ALBERS, R. (1909) Biochem. Z. 18, 40

FREEMAN, N.E., SMITHWICK, R.H., WHITE, J.C. (1934) Amer. J. Physiol. 107, 529

FRIEND, H. (1923) J. Biol. Chem. 57, 497

GADDUM, J.H., LEMBECK, F. (1949) Brit. J. Pharmacol. 4, 401

GADDUM, J.H., PEART, W.S., VOGT, M. (1949) J. Physiol. 108, 467

GADDUM, J.H., SCHILD, H. (1934) J. Physiol. 80, 9 P.

GAUTIER, C.L. (1912) C.R.Soc.Biol. 73, 564

GERARD, P., CORDIER, R., LISON, L. (1930) Bull. Histol. Tech. Micr. 7, 133

GIGON, A. (1923) Klin. Wschr. 2, 1670

GIORDANO, C., ZEGLIO, P. (1938) Z. Klin. Med. 135, 212

GIORDANO, C., ZEGLIO, P. (1939) Z. Klin. Med. 136, 213

GOHAR, H.A.F. (1934) J. Physiol. 80, 305

GOLDENBERG, M., PINES, K.L., BALDWIN, E. de F., GREENE, D.G., ROH, C.E. (1948) Amer. J. Med. 5, 792

GOLDENBERG, M., FABER, M., ALSTON, E.J., CHARGAFF, E.C. (1949) Science, 109, 534

GOODALL, Mc. Ch. (1950a) Acta Chem. Scand. 4, 550

GOODALL, Mc. Ch. (1950b) Nature (Lond.) 166, 738

GORDON, J.W. (1877) J. Anat. (Lond.) 11, 533

GREEN, D.E., RICHTER, D. (1937) Biochem. J. 31, 596

GROLLMAN, A. (1929) Amer. J. Physiol. 88, 432

HALE, W., SEIDELL, A. (1911) Amer. J. Pharm. 83, 551

HEALD, C.B., TUCKER, W.S. (1922) Proc.roy.Soc. London, 93, 281

HEILBRUNN, G., LIEBERT, E. (1939) Endocrinology 25, 354

HENDERSON, Y. (1905) Amer. J. Physiol. 14, 277

HENDERSON, Y., HAGGARD, H.W. (1925) Amer. J. Physiol. 73, 193

HENLE, J. (1865) Z. rat. med. 24, 142

HETENYI, G. (1926) Wien. Arch. inn. Med. 13, 95

HOFMANN, E. (1926) Krankheitsforschung 2, 295

HOKFELT, B. (1951) Acta physiol. Scand. 25, Suppl. 92

HOLTZ, P., CREDNER, K., KOEPP, W. (1942) Acta exp. Path. Pharmak. 200, 356

HOLTZ, P., CREDNER, K., KRONEBERG, G. (1947) Arch. exp. Path. Pharmak.

HOLZBAUER, M., VOGT, M. (1954) Brit. J. Pharmacol. 9, 249

HOUSSAY, B.A., LEWIS, J-T., MOLINELLI, E.A. (1924) C.R. Soc. Biol. 91, 1011

HOUSSAY, A., RAPELA, C.F. (1953) Arch. exp. Path. Pharmak. 219, 156

HUEBER, E.F. von (1940) Klin.Wschr. 19, 664

JAMES, W.O. (1948) Nature (Lond.) 161, 851

JENSEN, J. (1930) Amer. Heart J. 5, 763

JOHANNESSOHN, F. (1916) Biochem. J. 76, 377

JONES, M.S. (1939) Proc. roy. Soc. Med. 32, 958

JORGENSEN, K.S. (1945) Acta Pharmacol. 1, 225

JORGENSEN, K.S. (1948) Thesis, Kjobenhavn, A. Busch

JOSLIN, E.P. (1928) "The Treatment of Diabetes Mellitus," 4th Edit. Lea & Febiger, Philadelphia

KAHLSON, G., WERZ, R. (1930) Arch. exp. Path. Pharmak. 148, 173

KAHN, R.H. (1926) Pflugers Arch. ges. Physiol. 212, 54

KAINDL, F., EULER, U.S. von (1951) Amer. J. Physiol. 166, 284

KALAJA, L., SAVOLAINEN, H. (1941) Nord. Med. 12, 3562

KATZ, G.I. (1929) Odessky Med. J. 4, 207

KOBAYASHI (1935) Jap. J. Med. Sci. 8, 152

KOBRO, M. (1946) Acta med. Scand. 124, 511

KONSCHEGG, T., MONAUNI, J. (1936) Z.Klin.Med. 131, 99

KONSCHEGG, T., MONAUNI, J. (1938) Z.Klin.Med. 133, 632

KONZETT, H., WEISS, W. (1939) Arch. exp. Path. Pharmak. 193, 440

KOREFF, O., BENDEK, C. (1940) Cited in Chem. Abstr. 1943, 37, 4086

KRAUSS, L. (1908) Apothoker Ztg. 23, 701

KURE, K., OKINAKA, S., OHSHIMA, K., SHIMAMOTA, T., OKAMURA, D. (1936) Klin.Wschr. 15, 477

La BARRE, J., HOUSSA, P. (1932) C.R.Soc.Biol. 109, 967

LANDOIS, L. (1879)Lehrbuch der Physiologie des Menschen, Vienna, Urban u Schwarzenberg

LAUTER, S., BAUMANN, H. (1929) Dtsch. Arch. klin. Med. 163, 161

LEHMANN, G., MICHAELIS, H.F. (1942a) Arbeit physiologie, 12, 52

LEHMANN, G., MICHAELIS, H.F. (1942b) Arbeit physiologie, 12, 264

LOCKETT, M.F. (1954) Brit. J. Pharmacol. 9, 498

LOEW, O. (1918) Biochem. Z. 85, 295

LUDEMANN, H.H., FILBERT, M.G., CORNBLATH, M. (1955) J. appl. Physiol. 8, 59

LUND, A. (1949a) Acta Pharmacol. 5, 75

LUND, A. (1949b) Acta Pharmacol. 5, 121

LUND, A. (1949c) Acta Pharmacol. 5, 231

LUND, A. (1950) Acta Pharmacol. 6, 137

MAIWEG, H. (1922) Biochem. Z. 134, 292

MANGER, W.M., BALDES, E.J., FLOCK, E.V., BOLLMAN, J.L., BERKSON, J., JACOBS, M. (1953) Proc. Mayo Clin. 28, 526

MANGER, W.M., FLOCK, E.V., BERKSON, J., BOLLMAN, J.L., ROTH, G.M., BALDES, E.J., JACOBS, M. (1954) Circulation 10, 641

MEYTHALER, F., WOSSIDLO, K. (1935) Arch.exp.Path.Pharmak. 178, 320

MEYTHALER, F., LOBENHOFER, G., HAGGENMILLER, W. (1951) Dtsch. Arch. Klin. Med. 198,305

MIDDLETON, W.S., OATWAY, W.H.Jr. (1931) Amer.J.med.Sci. 181, 39

NATELSON, S., LUGOVOY, J.K., PINCUS, J.B. (1949) Arch. Biochem. 23, 157

NELSON, N. (1944) J. biol. Chem. 153, 375

NICKERSON, J.L., WARREN, J.V., BRANNON, E.S. (1947) J. clin. Invest. 26, 1

NOORDEN, C. von, ISAAC, S. (1927) "Die Zuckerkrankheit und Behandlung," 8th Edit. Julius Springer, Berlin

OLIVER, G., SCHAEFER, E.A. (1895) J. Physiol. 18, 230

OLMSTED, J.M.D., LOGAN, H.D. (1923) Amer.J.Physiol. 66, 437

ORRU (1929) Ann. Chim. appl., Roma, 19, 239

OUTSCHOORN, A.S. (1952) Brit.J.Pharmacol. 7, 605

PAGET, M. (1930) Bull. Sci. pharm. 37, 537

PAGET, M. (1931) Bull. Sci. pharm. 38, 77

PAGET, M., LOHEAC, P. (1928) J.Pharm. Chim., Paris, 8, 159

PARRY, G.H. (1825) "Collections from the published writings of the late C.H.Parry." Vol. 2. Underwood, London.

PARTINGTON, P.P. (1936) Amer. J. Physiol. 117, 55

PEKKARINEN, A. (1948) Acta physiol. Scand. 16, Suppl. 54

POLL, H. (1925) Med. klin. 21, 1717

PORAT, B. von (1946) Acta med. Scand. 123, 317

RAAB, W. (1941) Endocrinology, 28, 325

RAAB, W. (1943) Biochem. J. 37, 470

RAE (1930) Pharm. J. 125, 451

RANGIER, M. (1945) C.R.Soc.Biol. 220, 246

REINWIN, H. (1929) Dtsch. med. Wschr. 55, 951

RICCI, F., MILANI, A. (1925) Boll. Soc.Med.Chir., Paris, 1, 927

SAITO, S. (1929) Tohoku J. exp. Med. 12, 263

SANSONI (1930) Officina, 3, 323

SARFY, E. (1938) Z.physiol. Chem. 255, 271

SARFY, E. (1939) Z.physiol. Chem. 262, 87

SATTERTHWAITE, T.E. (1913) "Cardiovascular diseases." New York, Lemcke, Lemcke and Bucchner.

SCHLOSSBERG, T., SAWYER, M.E.M., BIXBY, E.M. (1933) Amer. J. Physiol. 104, 190

SCHLOSSMAN, N.H. (1927) Arch. exp. Path. Pharmak. 121, 160

SCOVILLE (1920) J.Ind.Eng.Chem. 12, 769

SHAW, F.H. (1938) Biochem.J. 32, 19

SHAW, F.H. (1941) Aust.J.exp.Biol.med.Sci. 19, 151

SHEPHERD, D.M., WEST, G.B. (1953) J.Physiol. 120, 15

SOMOGYI, M. (1945) J.biol.Chem. 160, 69

STARR, I. (1955) J.appl.Physiol. 8, 315

STARR, I. (1956) Personal communication.

STARR, I., RAWSON, A.J., SCHROEDER, H.A. , JOSEPH, N.R. (1939) Amer.J.Physiol. 127, 1

STAUB, H., KLINGLER, M. (1945) Helvet.physiol.pharmacol. Acta 3, 91

STEWART, G.N., ROGOFF, J.M. (1923) Amer.J.Physiol. 65, 342

STUBER, B., RUSSMANN, A., PROEBSTING, E.A. (1923a) J.Soc. Chem.Ind. 42, 739

STUBER, B., RUSSMANN, A., PROEBSTING, E.A. (1923b) Z.exp. Med. 32, 448

TAKAMINE, J. (1901) J.Physiol. 27, 26

TANNER, J.M. (1949) J.clin.Invest. 28, 567

THUNBERG, T. (1918) Scand.Arch.Phys. 35, 163

TIEGS, O.W. (1934) Proc.roy.Soc.London, 116, 351

TIETZ, E.B., BIRNBAUM, S.M. (1942) Amer.J.Psychiat. 99, 75

TIETZ, E.B., DORNHEGGEN, H., GOLDMAN,D. (1940) Endocrinology, 26, 641

TROTTER, C. (1877) J.Anat., London, 11, 755

TURNER, K.B. (1930) Amer. Heart J. 5, 671

UDENFRIEND, S., COOPER, J.R., CLARK, C.T., BAER, J.E. (1953) Science, 117, 663

VALK, A. de T. (1955) Personal Communication

VALK, A. de T. (1956) Personal Communication

VALK, A. de T., MILLAR, R.A. (1955) Unpublished observations, In Press

VALK, A. de T., PRICE, H.L. (1956) J.clin.Invest. In Press

VALK, A. de T., PRICE, H.L., MILLAR, R.A. (1955) Unpublished observations

VIALE, G. (1930) Cited in Chem.Abstr. (1931) 25, 2453

VIALE, G. (1933) Arch.int.Physiol. 36, 418

VIALE, G., CROCETTA, A. (1933) Cited in Chem.Abstr. 27, 5769

VOGT, M. (1947) J.Physiol. 106, 394

VOGT, M. (1952) Brit.J.Pharmacol. 7, 325

VULPIAN, A. (1856) C.R.Soc.Biol. 43, 663

WADA, M., KANOWOKA, Z. (1935) Tohoku J.exp.Med. 27, 1

WEIL-MALHERBE, H. (1953) Nature (Lond.) 172, 396

WEIL-MALHERBE, H. (1955a) Personal Communication

WEIL-MALHERBE, H. (1955b) J.ment.Sci. 101, 733

WEIL-MALHERBE, H., BONE, A.D. (1952a) Biochem.J. 51, 311

WEIL-MALHERBE, H., BONE, A.D. (1952b) J.ment.Sci. 98, 565

WEIL-MALHERBE, H., BONE, A.D. (1953) Lancet, 1, 974

WEIL-MALHERBE, H., BONE, A.D. (1954) J. Endocr. 11, 285

WEST, G.B. (1947) J. Physiol. 106, 426

WEST, G.B. (1951) Brit.J.Pharmacol. 6, 289

WHITEHORN, J.C. (1923) J.biol.Chem. 56, 751

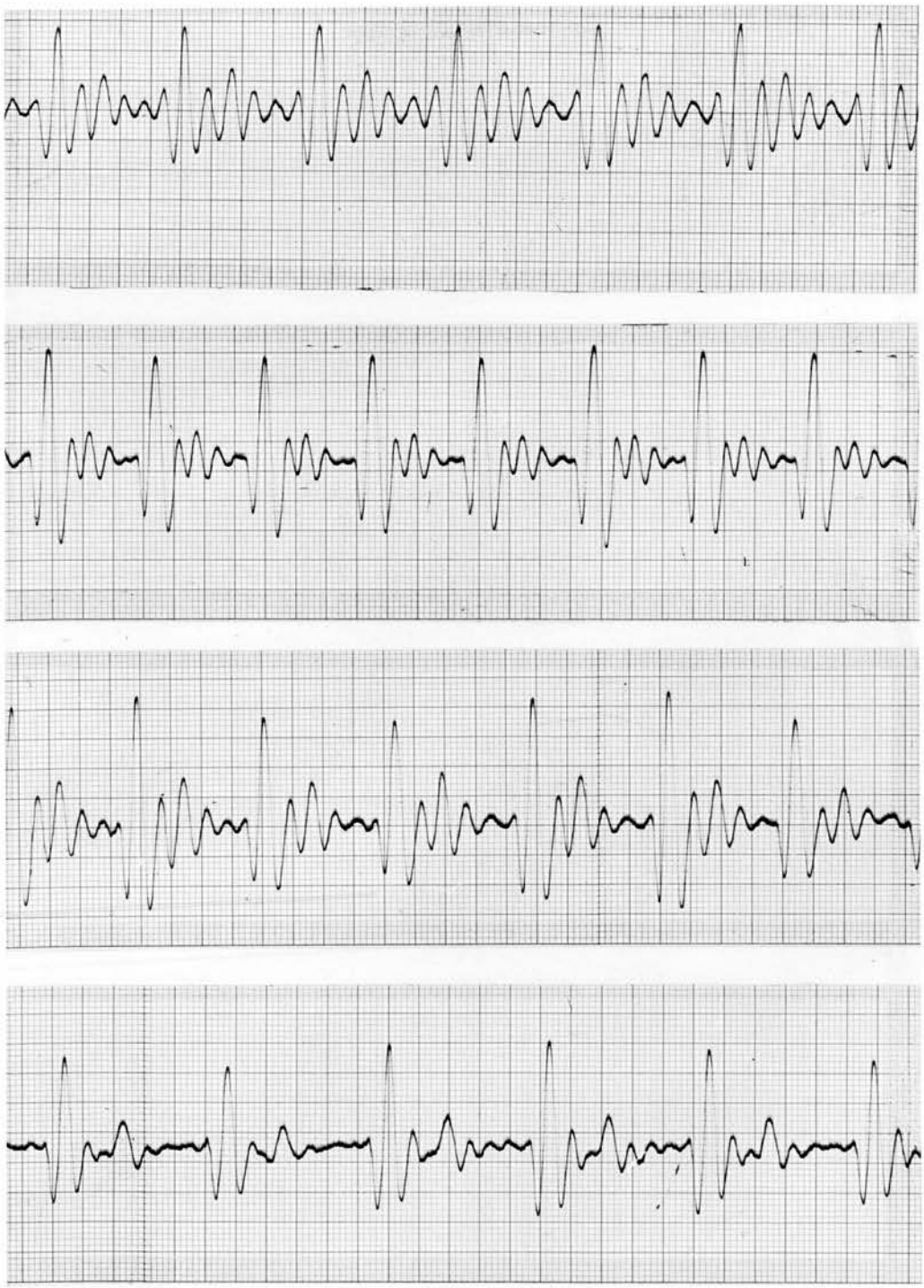
WHITEHORN, J.C. (1935) J.biol.Chem. 108, 633

WIESEL, H., SCHUR, J. (1907) Wien.klin.Wschr. 20, 699

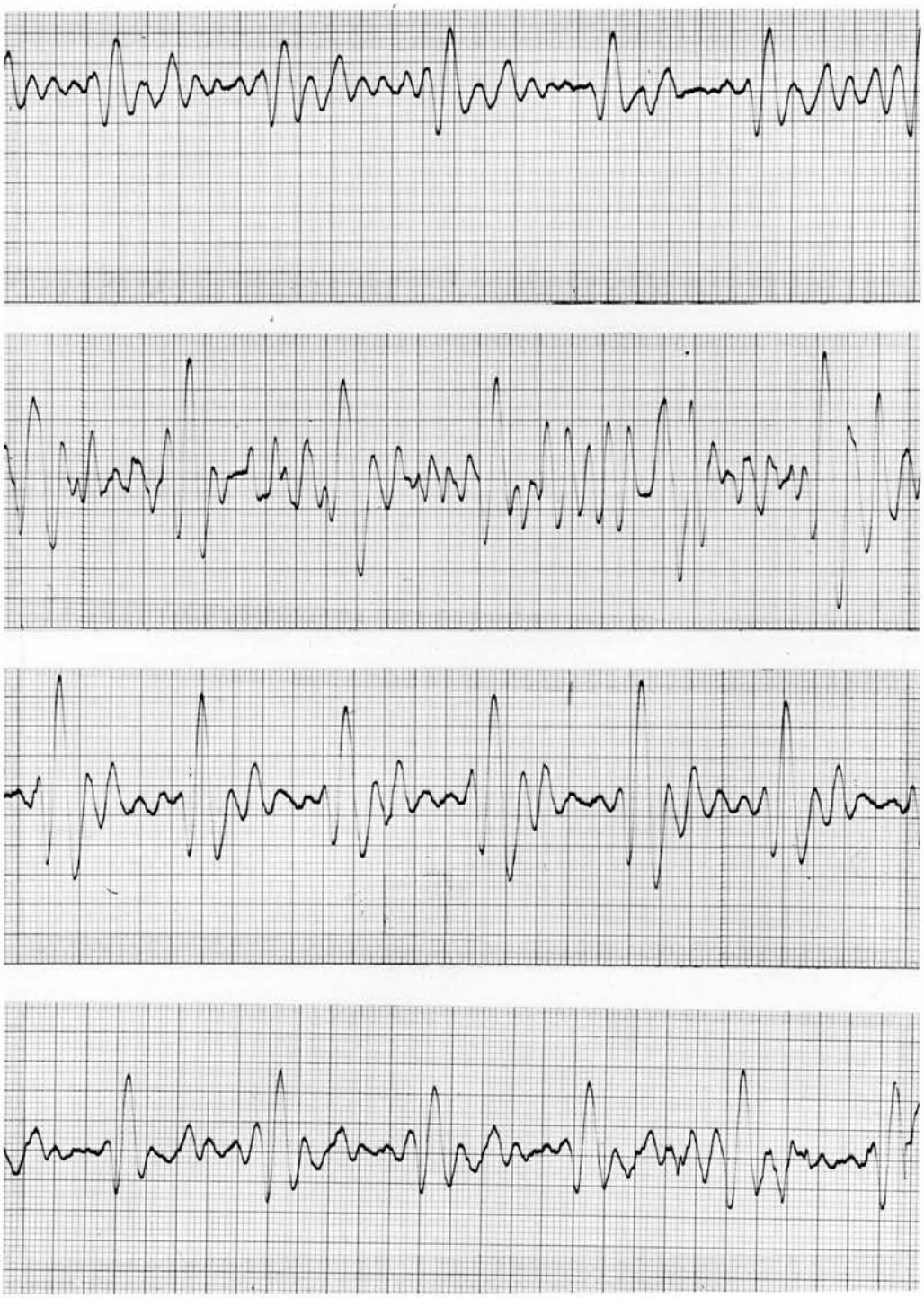
WILLIAMS, T.I. (1954) "The Elements of Chromatography," Blackie and Son, Glasgow.

YEN, T.J., AOMURA, T., INABA, T. (1933) Tohoku J.exp. Med. 21, 542

ZANFROGNINI, A. (1909) Dtsch.med.Wschr. 35, 1752

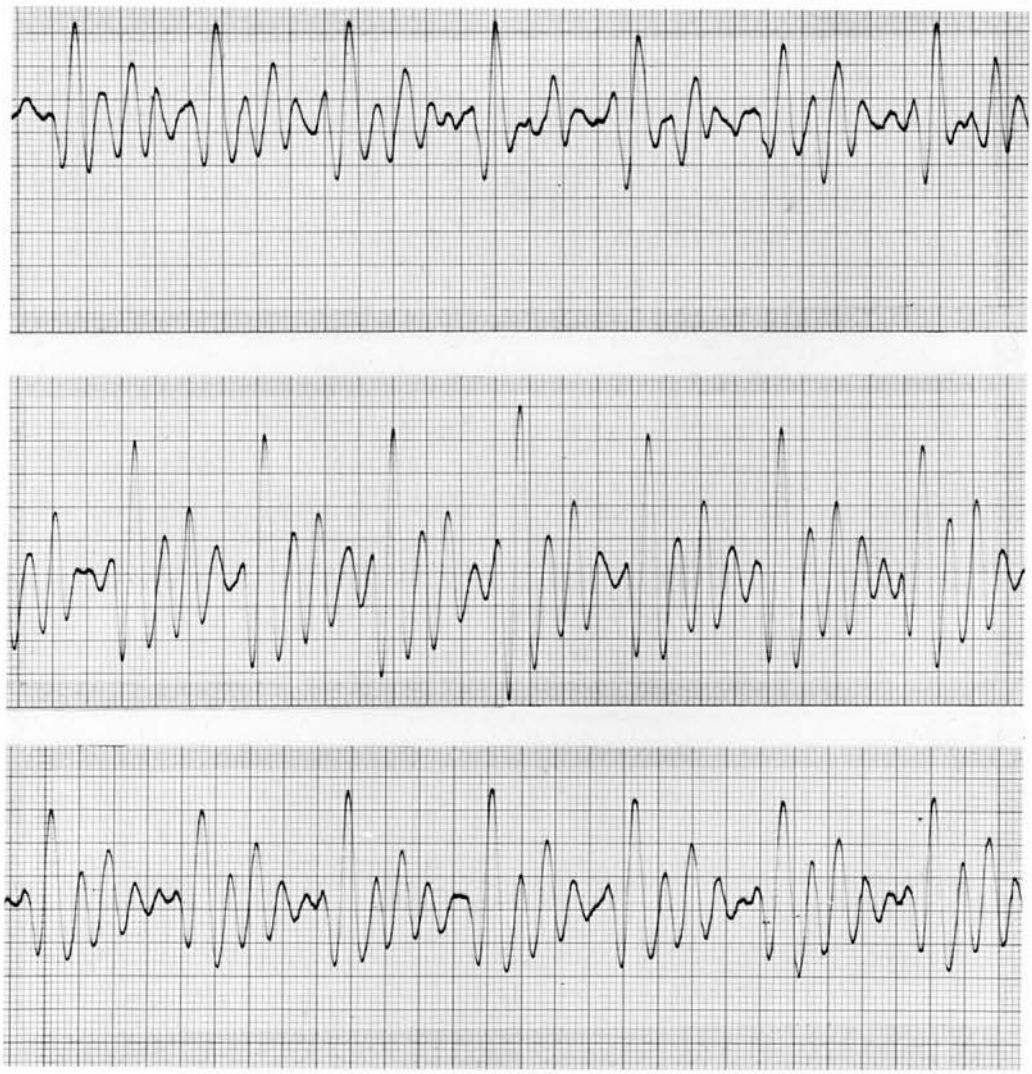


INSULIN - SUBJECT 3 (see page 137)



INSULIN - SUBJECT 4 (See page 137)

INSULIN – SUBJECT 5 (see page 137)



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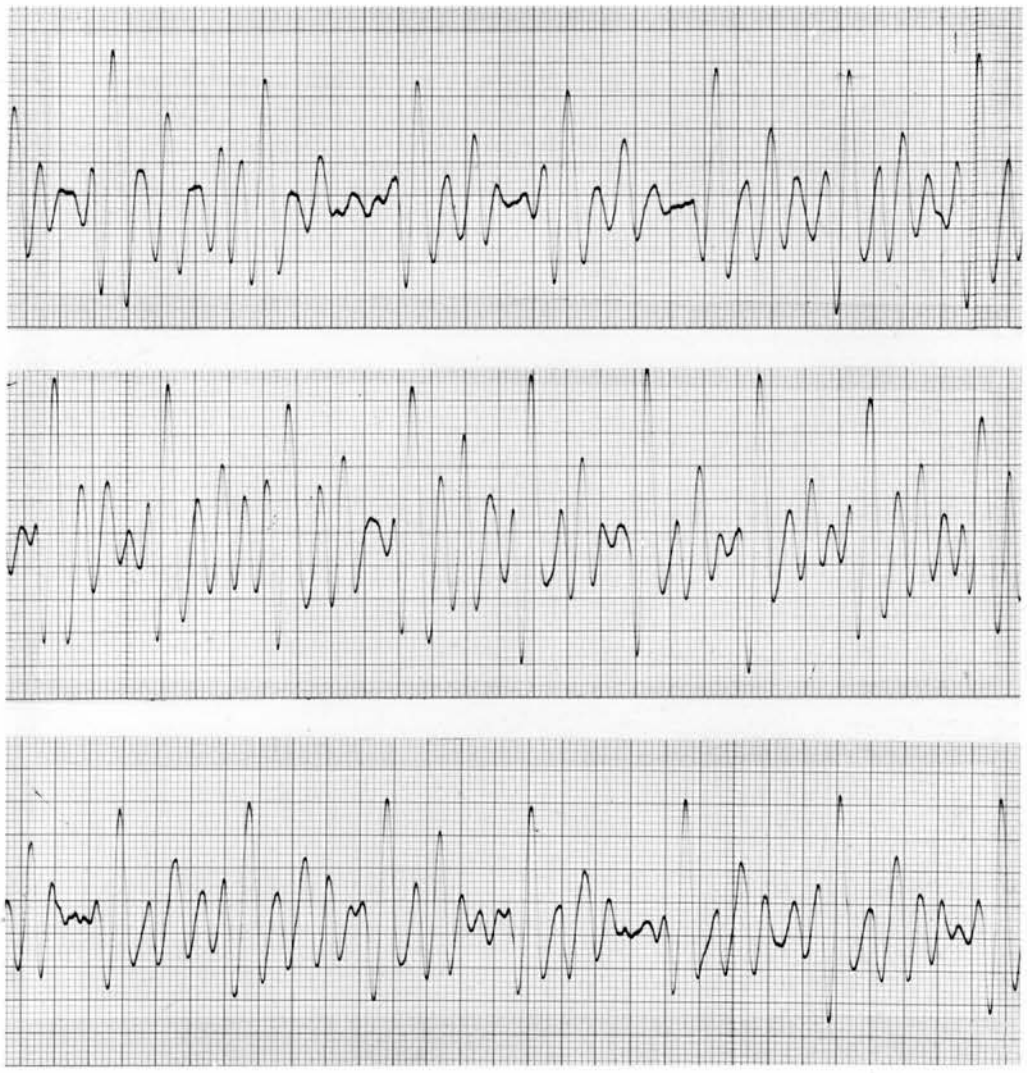

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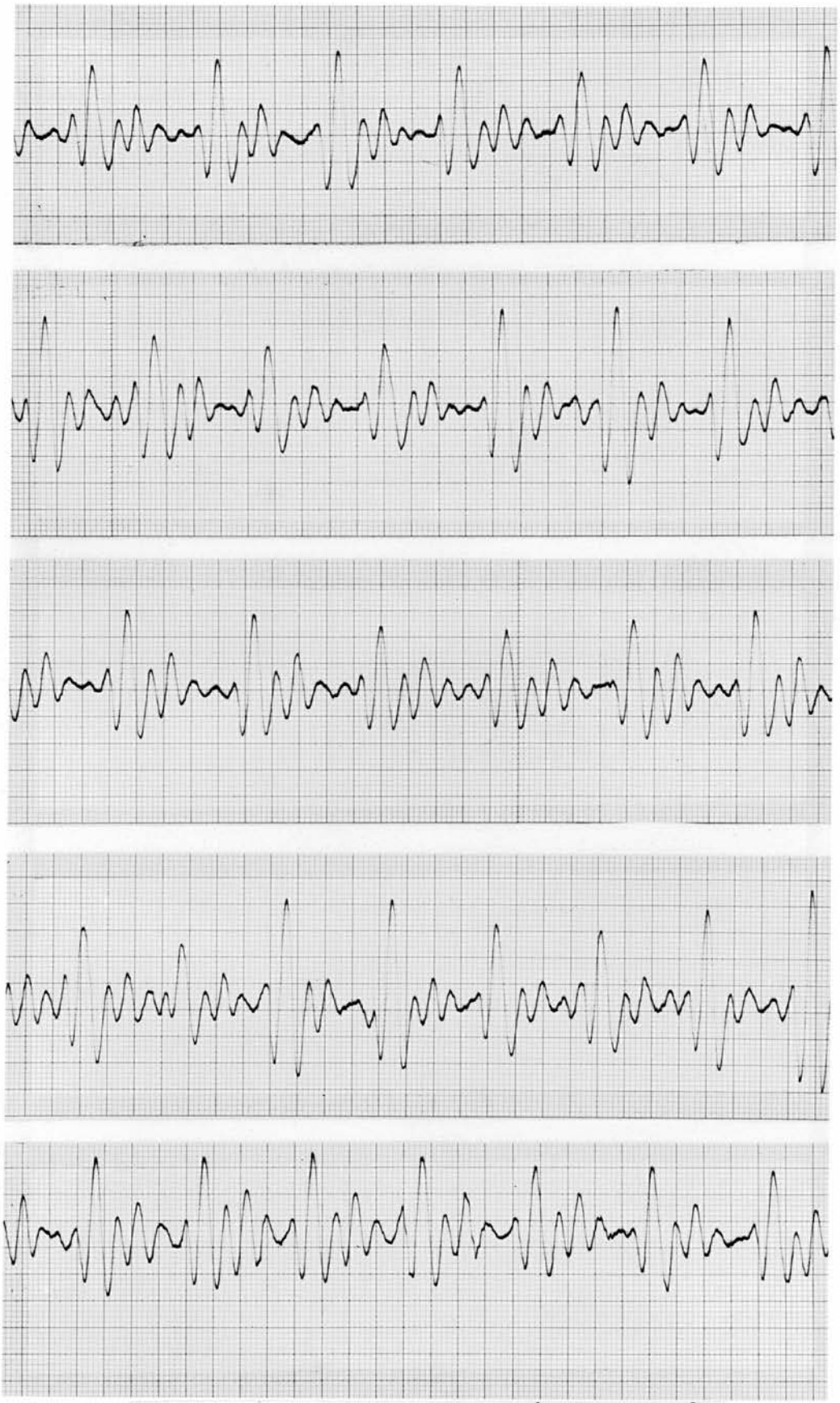

INFUSION 2 - SUBJECT 4 (see page 143)